



## Global epidemiology of non-typhoidal *Salmonella* infections in humans

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# Global epidemiology of non-typhoidal *Salmonella* infections in humans



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PhD Thesis  
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# LIST OF CONTENT

|   |             |
|---|-------------|
| <b>SUPERVISORS AND FUNDING.....</b>   | <b>i</b>    |
| <b>ACKNOWLEDGEMENTS.....</b>  | <b>iv</b>   |
| <b>LIST OF ORIGINAL ARTICLES .....</b>  | <b>v</b>    |
| <b>RESUMÉ.....</b>  | <b>vii</b>  |
| <b>SUMMARY.....</b>   | <b>x</b>    |
| <b>BACKGROUND, PURPOSE AND RESEARCH APPROACH .....</b>                                      | <b>xiii</b> |
| <b>INTRODUCTION.....</b>  | <b>1</b>    |
| <b>GLOBAL DISTRIBUTION OF SEROVARS AND TRENDS IN HUMANS.....</b>                            | <b>2</b>    |
| <b>HUMAN EPIDEMIOLOGY .....</b>   | <b>7</b>    |
| <i>The public health burden of Salmonella infections.....</i>                               | <i>7</i>    |
| <i>Symptoms and human infections .....</i>  | <i>9</i>    |
| <i>Age, season, and risk factors.....</i>   | <i>10</i>   |
| <i>Antimicrobial treatment and antimicrobial resistance .....</i>                           | <i>11</i>   |
| <b>MAIN RESERVOIRS .....</b>  | <b>13</b>   |
| <i>Host adapted and host restricted serovars.....</i>                                       | <i>14</i>   |
| <i>Host non-specific serovars .....</i>   | <i>15</i>   |
| <b>TRANSMISSION – LOCAL AND GLOBAL .....</b>  | <b>20</b>   |
| <i>Primary routes of transmission.....</i>  | <i>20</i>   |
| <i>Source attribution.....</i>  | <i>22</i>   |
| <i>International trade of food and animals .....</i>  | <i>23</i>   |
| <i>International travel .....</i>   | <i>24</i>   |
| <i>International adoptions .....</i>  | <i>25</i>   |
| <b>SURVEILLANCE .....</b>   | <b>27</b>   |
| <i>Surveillance.....</i>  | <i>27</i>   |
| <i>Surveillance methods.....</i>  | <i>28</i>   |
| <i>Surveillance programmes.....</i>   | <i>30</i>   |
| <i>A harmonised multi-national integrated laboratory-based surveillance programme .....</i> | <i>32</i>   |
| <i>Outbreak surveillance programmes .....</i>   | <i>34</i>   |
| <b>CONTROL, INTERVENTION, AND PREVENTION .....</b>  | <b>35</b>   |
| <i>Analysis of surveillance data.....</i>   | <i>35</i>   |

*Host specific control programmes and interventions* .....36

*Serovar specific control programmes and interventions* .....38

*Prevention by vaccination* .....38

**FUTURE PREDICTIONS AND PERSPECTIVES** .....39

**CONCLUSIONS AND RECOMMENDATIONS** .....41

**REFERENCES** .....42

**ARTICLES** .....59

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## LIST OF ORIGINAL ARTICLES

The thesis is structured as a review of the global epidemiology of non-typhoidal *Salmonella* (NTS) infections in humans and six articles that are published or submitted for publication in peer reviewed international journals. Articles are referred in the text by roman letters and marked in **bold** typeface.

- I.     **Hendriksen RS**, Mikoleit M, Carlson VP, Karlslose S, Vieira AR, Jensen AB, Seyfarth AM, Delong SM, Weill FX, Lo Fo Wong DM, Angulo FJ, Wegener HC, Aarestrup FM. WHO Global Salm-Surv External Quality Assurance System (EQAS) for serotyping of *Salmonella* isolates, 2000 - 2007. J Clin Microbiol. 2009 Sep; 47 (9): 2729-36.
  
- II.    **Hendriksen RS**, Vieira AR, Karlslose S, Mikoleit M, Lo Fo Wong DMA, Jensen AB, Delong SM, Wegener HC, Angulo FJ, Aarestrup FM.  
Global monitoring of *Salmonella* serovar distribution based on quality assured data from the WHO Global Salm-Surv Country Data Bank; 2001 – 2007. To be submitted to: Foodborne Pathog Dis.
  
- III.   **Hendriksen RS**, Bangtrakulnonth A, Pulsrikarn C, Pornreongwong S, Hasman H, Song SW, Aarestrup FM.  
Antimicrobial resistance and molecular epidemiology of *Salmonella* Rissen from animals, food products, and patients in Thailand and Denmark. Foodborne Pathog Dis. 2008 Oct; 5 (5): 605-19.
  
- IV.    **Hendriksen RS**, Mikoleit M, Kornschöber C, Rickert RL, Van Duyn S, Kjelsø C, Hasman H, Cormican M, Mevius D, Threlfall EJ, Angulo FJ, Aarestrup FM.  
Emergence of Multidrug-Resistant *Salmonella* Concord Infections in Europe and the United States in Children Adopted From Ethiopia, 2003–2007. Pediatr Infect Dis J. 2009 Sep; 28 (9): 814-818.



- V. Sirichote P, Hasman H, Pulsrikarn C, Schönheyder HC, Samulionienė J, Pornreongwong S, Bangtrakulnonth A, Aarestrup FM, **Hendriksen RS**.  
Molecular characterization of extended spectrum cephalosporinases (ESC) producing *Salmonella* Choleraesuis from patients in Thailand and Denmark. *Submitted to: J Clin Microbiol*.
- VI. **Hendriksen RS**, Bangtrakulnonth A, Pulsrikarn C, Pornreongwong S, Noppornphan G, Emborg HD, Aarestrup FM.  
Risk Factors and Epidemiology of the Ten Most Common *Salmonella* Serovars from Patients in Thailand: 2002–2007. *Foodborne Pathog Dis*. 2009 Oct; 6 (8): 1009-19.

## RESUMÉ

Globalisering, internationale rejser og handel øger hastigt den globale udbredelse og overførsel af levnedsmiddelbårne patogener imellem lande. Allerede i dag er mere end halvdelen af alle humane *Salmonella* infektioner i Danmark forårsaget af internationale rejser og af importerede fødevarer. I store dele af verden har *Salmonella enterica* serovar Enteritidis og *S. Typhimurium* indtil nu tiltrukket sig mest opmærksomhed. Dog er andre *Salmonella* serotyper ofte mere udbredt i enkelte lande og resultere i flere alvorlige infektioner og udbrud. Det er således vigtigt at undersøge epidemiologien af *Salmonella* globalt.

Det er vigtigt, at data fra forskellige lande er sammenlignelige. Vi har vurderet kvaliteten af *Salmonella* overvågningsdata fra laboratorier i hele verden samt deres evne til at serotype baseret på deltagelse i WHO's Globale Foodborne Infections Network (GFN) External Quality Assurance System (EQAS). Syv EQAS-runder blev gennemført mellem 2000 og 2007. Deltagende laboratorier indsendte serotypningsresultater for otte *Salmonella* isolater i hver runde. I alt deltog 249 laboratorier i 96 lande i mindst én EQAS runde. Totalt set indsendte 76% af de deltagende laboratorier data for alle otte stammer og 82% af stammerne var korrekt serotypet.

Præstationsmæssigt blev der observeret regionale forskelle blandt laboratorier fra Centralasien og Mellemøsten, som udførte testene mindre godt sammenlignet med de øvrige regioner. Fejl, som resulterede i forkert identifikation af serotypen, var typisk forårsaget af vanskeligheder med at detektere andenfasen af flagel antigenet eller differentiering indenfor antigen komplekser. Nogle af disse fejl er sandsynligvis relateret til kvaliteten af den antisera, som har været til rådighed (I). Baseret på data fra GFN Country Data Bank (CDB) i årene 2001 til 2007 sammenfattede vi den globale fordeling af *Salmonella* serotyper i udvalgte lande for at afdække de regionale og globale tendenser og mønstre i forekomsten af *Salmonella* serotyper. Sammendraget var baseret på kvalitetssikrede data fra 37 lande, som alle har bestået kvalitetssikringskravet til GFN EQAS. Vi har fundet betydelige forskelle imellem de mest almindeligt isolerede serotyper i forskellige geografiske områder, hvorimod flere lignende serotyper blev rapporteret i lande fra det samme område. Der blev også observeret en faldende tendens til at isolere og serotype i de lande som indgår i denne undersøgelse. Et par serotyper dominerede i hele verden, men var til stede med forskellig hyppighed i forskellige regioner. Vi bemærkede interessant nok, at globalt set er den relative betydning af *S. Enteritidis* og *S. Typhimurium* faldende, mens andre serotyper såsom *S. Typhi*, *S. Infantis*, *S. Hadar*, *S. Newport*, *S. Virchow*, *S. Agona* og andre serovars er stigende (II).

En række "case-studies" blev udført for at undersøge den globale spredning af *Salmonella*. Den genetiske diversitet og resistensprofilen blev undersøgt på 112 *S. Rissen* isolater fundet blandt mennesker, fødevarer og dyr i Danmark og Thailand. Derudover blev risikofaktorer såsom "at rejse" og "indtagelse af bestemte fødevarer" analyseret og evalueret. Der blev i alt observeret 63 unikke *Xba*I pulsed field gel elektroforese (PFGE) mønstre, hvoraf det dominerende mønster blev delt af 22 stammer. Der blev observeret et begrænset niveau af antibiotikaresistens i de danske stammer, hvorimod der blev observeret en højere grad af resistens i stammer fra Thailand. Statistiske analyser og molekylær subtypning identificerede kombinationen af "rejse til Thailand" og "konsumering af importerede svin / svinekødsprodukter" samt "konsumering af svin / svinekød produceret i Danmark", som risikofaktorer for *S. Rissen*-infektioner blandt danske patienter (III).

Der er blevet rapporteret om multiresistente *S. Concord* infektioner blandt børn adopteret fra Etiopien til Østrig, Danmark, England (og Wales), Irland, Holland og USA. Vi interviewede patienter, karakteriserede isolater og indsamlede oplysninger om adoptioner fra Etiopien for at vurdere konsekvenserne for folkesundheden. Isolaterne er blevet subtyperet ved brug af PFGE og resistensprofiler; specifikke resistens-gener er blevet karakteriseret. Adoptionsstatus var tilgængelig for 44 patienter <3 år, hvoraf 98% var blevet adopteret fra Etiopien. De adopterede børn kom fra forskellige børnehjem i Etiopien. På de besøgte børnehjem var der dårlig hygiejne og sanitære forhold samt hyppig brug af antibiotika. Der var 53 PFGE mønstre blandt 64 *S. Concord* isolater. Der blev udført resistensbestemmelser på 43 isolater, hvoraf 81% var multiresistente ( $\geq 3$  stoffer). De multi-resistente isolater var fra etiopiske adoptivbørn og var resistente over for tredje og fjerde generations cephalosporiner. Herudover havde 14% nedsat følsomhed over for ciprofloxacin (IV).

Vi har også karakteriseret 24 udvidet spektrum cefalosporinase-producerende isolater af *S. Choleraesuis* fra thailandske og danske patienter. Treogtyve af isolaterne var fra thailandske patienter fra år 2003, 2007 eller 2008. Yderligere var et af isolaterne fra en dansker, som havde været rejsende i Thailand. De 13 af isolaterne var fra blodprøver. MIC-bestemmelse, micro-array, PCR, plasmid-profilering og replikon-typning har afsløret forekomst af multiresistente isolater med plasmider der varierer i størrelse fra 75 -200 kb indeholdende enten *bla*<sub>CMY-2</sub> inklusiv *incA/C* eller *bla*<sub>CTX-M-14</sub> inklusiv *incFIIA* / *incFrepB*. RFLP og replikon-typning fordelte isolaterne i fire adskilte grupper. PFGE afslørede 16 unikke mønstre og fem grupper. Isolatet fra den danske

patient var identisk med to kliniske isolater fra Thailand. Undersøgelsen viste fremkomsten af *bla*<sub>CTX-M-14</sub> genet blandt adskillige kloner af *S. Choleraesuis*. Adskillige plasmider blev identificeret indeholdende op til to forskellige udvidet spektrum cefalosporinase gener og fire forskellige replikonner. En rejse-associeret spredning blev bekræftet (V).

De fleste undersøgelser af epidemiologien af *Salmonella* er fra lande, hvor *S. Enteritidis* og *S. Typhimurium* var dominerende. Der ses dog et andet mønster i Thailand, hvor vi har foretaget et retrospektivt observationsstudie fra 2002 til og med 2007 for at vurdere epidemiologiske tendenser og risikofaktorer forbundet med de ti mest almindelige *Salmonella* serotyper isoleret fra mennesker. Der blev inkluderet i alt 11.656 *Salmonella* isolater i undersøgelsen dækkende alle seks år. De fleste af isolater var fra patienter <5 år (33%), isoleret i juni (13%), fra fæces (82%) og fra Bangkok (27%). Statistiske analyser viste, at *S. Enteritidis* og *S. Choleraesuis* blev isoleret fra blod med en højere frekvens end andre ikke-typhoide serotyper. Der var en tendens til at begge serotyper blev isoleret fra patienter ældre end 5 år. *S. Choleraesuis* blev fundet med en højere frekvens i patienter fra Bangkok og den centrale region, mens *S. Enteritidis* overvejende blev fundet i patienter fra den sydlige region. Undersøgelsen viser også i forhold til tidligere undersøgelser et skift i forekomsten af de mest almindelige *Salmonella* serotyper forbundet med humane infektioner i Thailand. Der var blandt andet en stigning i humane infektioner med *S. Stanley*, *S. Corvallis*, og *S. Choleraesuis*, som tidligere har været forbundet med svin. Yderligere var der et fald i infektioner forårsaget af *S. Weltevreden* og *S. Anatum* (VI).

Samlet set har denne Ph.D. afhandling vurderet kvaliteten af *Salmonella* serotypning foretaget på de nationale referencelaboratorier samt brugt disse data til at beskrive udviklingen i den globale distribution af *Salmonella*. Desuden har den vist forbindelser imellem forskellige reservoirer og kilder til salmonellosis hos mennesker i forskellige områder af verden og anvendt thailandske overvågningsdata til at opstille risikofaktorer for salmonellose hos mennesker i Thailand. Den er kommet med flere anbefalinger til aktioner, hvad angår kontrol samt forebyggelse af infektioner i mennesker.

## SUMMARY

Globalization, international travel, and trade among countries facilitate the rapid global spread and transmission of food borne pathogens. Currently, more than half of all human *Salmonella* infections in Denmark result from international travel and consumption of imported food.

Worldwide, *Salmonella enterica* serovar Enteritidis and *S. Typhimurium* cause the majority of human clinical cases. However, other *Salmonella* serovars are often more prevalent in specific countries, and result in more severe infections and outcome. It is, thus, important to study *Salmonella* epidemiology globally.

It is essential that data from different countries are comparable. We assessed the quality of the *Salmonella* surveillance data worldwide, and the laboratories ability to accurately determine serotype, based on participation in the WHO Global Foodborne Infections Network (GFN) External Quality Assurance System (EQAS) for serotyping of *Salmonella*. Seven EQAS iterations were conducted between 2000 and 2007. In each iteration, participating laboratories submitted serotyping results for eight *Salmonella* isolates. A total of 249 laboratories in 96 countries participated in at least one EQAS iteration. Cumulatively, 76% of participating laboratories submitted data for all eight strains and 82% of strains were correctly serotyped. Regional variations in performance were observed, with higher error rates in laboratories in Central Asia and the Middle East compared with other regions. Errors that resulted in incorrect serovar determinations were usually caused by difficulties either in the detection of the phase II flagellar antigen or differentiation within antigen complexes. Some of these errors likely were related to the quality of the antisera available (I).

We summarised the global distribution of *Salmonella* serovars of selected countries, based on 2001-2007 data from GFN Country Data Bank (CDB) to uncover regional and global trends in the occurrence of *Salmonella* serovars. The summary was based on quality-assured data from 37 countries that passed the quality assurance threshold of the GFN EQAS. We found considerable differences in the most commonly isolated serovars in various geographical regions, and more similar serovars prevalences in countries from the same region. We observed a tendency among countries included in this study to isolate and serotype less compared to previous years. A few serovars predominated worldwide, but were present with different frequencies in different regions. Interestingly, we observed that the relative importance of *S. Enteritidis* and *S.*

Typhimurium is decreasing globally, while other serovars such as *S. Typhi*, *S. Infantis*, *S. Hadar*, *S. Newport*, *S. Virchow*, *S. Agona* and other serovars are increasing (II).

A number of case studies were conducted to investigate the global spread of *Salmonella*. The genetic diversity and antimicrobial resistance of 112 *S. Rissen* isolates recovered from humans, food products and animals in Denmark and Thailand were examined. Additionally, risk factors due to travel and consumption of specific food products were analyzed and evaluated. A total of 63 unique *XbaI* pulsed field gel electrophoresis (PFGE) patterns were observed. The predominant pattern was shared by 22 strains. Limited antimicrobial resistance was observed in the Danish strains, whereas a higher degree of resistance was observed in strains originating from Thailand. Statistical analysis and molecular subtyping identified the combination of “travel to Thailand” and “consumption of imported pig / pork products” as well “consumption of as pig / pork products produced in Denmark” as risk factors for *S. Rissen* infection among the Danish patients (III).

Multidrug-resistant *S. Concord* infections have been reported from children adopted from Ethiopia to Austria, Denmark, England (and Wales), Ireland, the Netherlands and the United States. We interviewed patients, characterized the isolates, and gathered information about adoptions from Ethiopia to assess public health implications. Isolates were subtyped by PFGE and antimicrobial susceptibility; specific antimicrobial resistance genes were characterized. Adoption status was known for 44 patients  $\leq 3$  years of age; 98% were adopted from Ethiopia. The children adopted from Ethiopia were from several orphanages; visited orphanages had poor hygiene and sanitation and frequent use of antimicrobial agents. Sixty-four *S. Concord* isolates yielded 53 PFGE patterns. Antimicrobial susceptibility was performed on 43 isolates; 81% were multidrug-resistant ( $\geq 3$  agents). Multidrug-resistant isolates were from Ethiopian adoptees and were resistant to third and fourth generation cephalosporins, with 14% showing decreased susceptibility to ciprofloxacin (IV).

We also characterized 24 extended spectrum cephalosporinases (ESC) producing isolates of *S. Choleraesuis* recovered from patients in Thailand and Denmark. Twenty-three isolates were recovered from Thai patients in 2003, 2007, or 2008 and one isolate was recovered from a Danish traveler to Thailand, 13 of which were blood culture isolates. MIC determination, micro-array, PCR, plasmid profiling and replicon typing revealed the presence of multi-drug resistant isolates harboring either *bla*<sub>CMY-2</sub> containing *incA/C* or *bla*<sub>CTX-M-14</sub> containing *incFIIA* / *incFrepB*

plasmids ranging in size from 75–200 kb. The RFLP and replicon typing clustered the isolates into four distinct groups. PFGE revealed 16 unique patterns and five clusters. The isolate from the Danish patient was indistinguishable from two Thai clinical isolates. This study revealed the emergence of the *bla*<sub>CTX-M-14</sub> gene among several clones of *S. Choleraesuis*. Numerous plasmids were identified containing up to two different ESC genes and four distinct replicons. A “travel associated” spread was confirmed (V).

Most studies of *Salmonella* epidemiology have been in countries where *S. Enteritidis* and *S. Typhimurium* predominated. In Thailand, a different pattern is observed. We conducted a retrospective observational study to assess epidemiological trends and risk factors associated with the ten most common *Salmonella* serovars isolated from humans in Thailand between 2002 and 2007. A total of 11,656 *Salmonella* isolates covering all six years were included in the study. Most isolates were from patients <5 years (33%), isolated during June (13%), recovered from stool (82%) and from Bangkok (27%). Statistical analysis revealed that *S. Enteritidis* and *S. Choleraesuis* were recovered from blood with a higher frequency than other non-typhoidal serovars. While both serovars tended to be isolated from patients older than 5 years; *S. Choleraesuis* was recovered with a higher frequency from patients in Bangkok and the Central Region, whereas *S. Enteritidis* was recovered predominantly from patients in the Southern Region. This study also indicates a shift in prevalence of the most common *Salmonella* serovars responsible for human infections in Thailand compared to previous studies. Notably, there was an increase in human infections with *S. Stanley*, *S. Corvallis*, and *S. Choleraesuis* - three serovars which previously have been associated with swine - and a decrease in infections due to *S. Weltevreden* and *S. Anatum* (VI).

Overall, this Ph.D. thesis has assessed the quality of *Salmonella* serotyping conducted in national reference laboratories and used these data to describe trends in the global distribution of *Salmonella*. In addition, it has revealed links between different reservoirs and sources to human salmonellosis in different areas of the world and used Thai surveillance data to set up risk factors for human salmonellosis in Thailand. In several cases, this work has resulted in recommendations to help control and prevent infections in humans.

## BACKGROUND

Today, we are all residents of a global village. The expanding trade of food and livestock, and increased human travel and migration are means of spreading infectious disease irrespective of national borders. This makes infectious disease control and food safety important for all countries. In Denmark, it is expected that in a few years, around 2/3 of all food products will originate from other countries. Already today, more than half of all human *Salmonella* infections in Denmark are caused by international travel and consumption of imported food.

In addition, the majority of the *Salmonella* isolates causing human infections in Denmark by consumption of imported food products are resistant to multiple antimicrobials, which has increased in many countries that export foods to Denmark.

In Europe and North America, *S. Enteritidis* and *S. Typhimurium* have, up until now, drawn most attention. However, other *Salmonella* serovars are often more prevalent in other parts of the world and result in more severe infections with higher morbidity. Thus, there is an urgent need to further investigate and elucidate the occurrence, international spread, and global epidemiology of *Salmonella* serovars and specific clones so that evidence-based interventions can be taken worldwide.

## PURPOSE

The purpose of the PhD project was to study the global epidemiology of NTS infections in humans. The term “epidemiology” is defined in the traditional way as the study of the occurrence and distribution of a disease in a population and the factors which influence disease occurrence.

The PhD project integrated conventional and molecular microbiology used to characterise isolates with epidemiological and statistical tools needed to estimate trends and risk factors.

## RESEARCH APPROACH

The projects were derived from the activities of the WHO Global Foodborne Infections Network (GFN), a network of institutions building capacity for laboratory-based surveillance of foodborne pathogens and disease (<http://www.who.int/salmsurv/en/>), to assess the global distribution of



*Salmonella* serovars, investigate examples of international spread and identify regional and local risk factors for infection.

The specific studies conducted during this PhD project focused on the following objectives:

1. To assess the quality of the *Salmonella* surveillance data worldwide and the laboratories ability to serotype based on participation in the GFN EQAS.
2. To estimate the global distribution and trends of *Salmonella* serovars from the GFN Country Data Bank (CDB). CDB data reliability was based on results from the GFN EQAS.
3. To investigate the spread of *S. Rissen* caused by international travel to Thailand and imported food from Spain and Germany.
4. To investigate the spread of *S. Concord* to Europe and United States through adopted children from Ethiopia.
5. To characterise the extended spectrum cephalosporinases genes of the invasive serovar *S. Choleraesuis*.
6. To identify risk factors in the epidemiology of *Salmonella* serovars in Thai patients and to use these findings to recommend strategies for control and prevention.

## INTRODUCTION

*Salmonella* is a genus of rod-shaped, Gram-negative, oxidase negative, non-spore forming, predominantly motile (peritrichous) bacteria belonging to the family *Enterobacteriaceae*. *Salmonella* are approximately 0.7 to 1.5 µm wide and 2.0 to 5.0 µm in length (Giannella *et al.*, 1996). The bacterium ferments glucose and usually with production of gas. In addition, they are able to grow in a minimal media containing glucose as the sole carbon energy source and ammonium ion as a nitrogen source (prototrophic). Most serovars are phenotypically identified by urea hydrolysis, the absence of tryptophan deaminase, non-lactose fermentation, the production of hydrogen sulphide (H<sub>2</sub>S), decarboxylate lysine and ornithine and growth on Simmons citrate agar (Grimont *et al.*, 2000).

The genus *Salmonella*, first known as *Salmonella choleraesuis*, was initially discovered in 1886 by Theobald Smith and Daniel Elmer Salmon. The discovery of the genus originated from the work on swine fever (hog cholera) by Theobald Smith and he named the genus after his supervisor at the U.S. Department of Agriculture (USDA), Daniel E. Salmon (Grimont *et al.*, 2000).

In the late 19<sup>th</sup> century, serological tests utilizing agglutination with antiserum were developed, and new serovars were discovered and named after either clinical conditions or hosts, e.g.

*Salmonella enteritidis*, *Salmonella abortusovis*, *Salmonella gallinarum*, *Salmonella bovis*, *Salmonella typhimurium* (Grimont *et al.*, 2000).

In 1926, Bruce White developed the analysis of somatic and flagella antigens, which in 1961 was expanded by Fritz Kauffman to distinguish more than 2000 serovars. In 1980, the *Salmonella* nomenclature of today (The Kauffman-White Scheme) was proposed, and is currently maintained by the World Health Organization (WHO) Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute, Paris, France (Grimont *et al.*, 2000; Grimont *et al.*, 2007).

Currently, *Salmonella* consists of 2,579 different serovars divided into two species – *Salmonella enterica* (n=2,557) replacing the old name *Salmonella choleraesuis* (Hohmann *et al.*, 2001) and *Salmonella bongori* (n=22) (Grimont *et al.*, 2007). The species, *Salmonella enterica* is further divided into six subspecies - *Salmonella enterica* subsp. *enterica* (I), *Salmonella enterica* subsp. *salamae* (II), *Salmonella enterica* subsp. *arizonae* (IIIa), *Salmonella enterica* subsp. *diarizonae* (IIIb), *Salmonella enterica* subsp. *houtenae* (IV), and *Salmonella enterica* subsp. *indica* (VI). Serovars of *Salmonella enterica* subsp. *enterica* (I), are primarily named by the geographical

origin such as *S. Amsterdam*, *S. Panama*, and *S. Derby* whereas the serovars of the remaining five subspecies all are named by antigenic formula (Grimont *et al.*, 2000; Grimont *et al.*, 2007).

## GLOBAL DISTRIBUTION OF SEROVARS AND TRENDS IN HUMANS

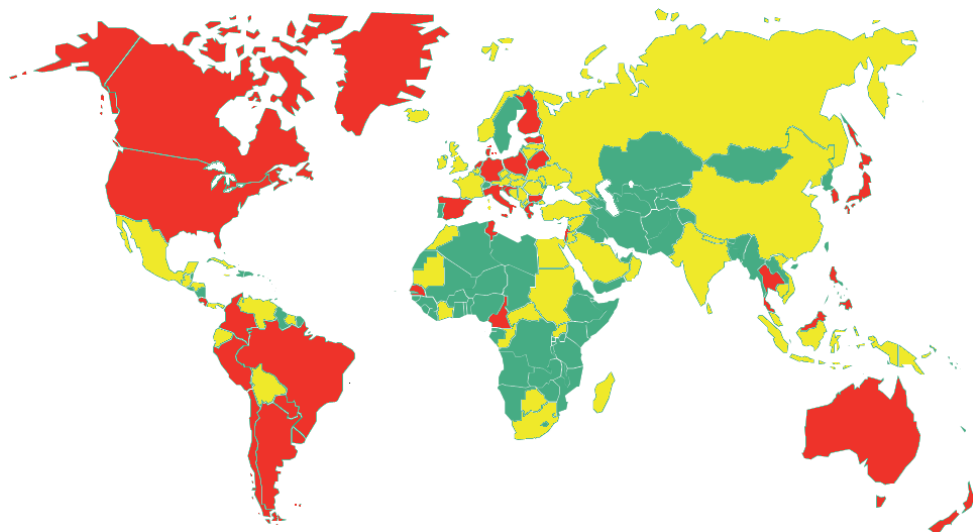
*Salmonella* serotyping still serves as the predominately used surveillance tool for detection of outbreaks and corresponding sources, to monitor trends over time, and attribute different food and animal reservoirs to human infections. Despite this, there is today only limited knowledge of the global distribution of *Salmonella* serovars in humans. In the last decade, some countries have collected annual prevalence data on serovar distribution among humans, but very few publications have summarised the global distribution of the serovars responsible for human infections and further analysed the data (Herikstad *et al.*, 2002; Galanis *et al.*, 2006; II). An equally important feature for surveillance is quality assurance and quality control, which is necessary to ensure reliable data. Only a small number of international quality assurance systems exist to evaluate the quality of the serotyping conducted worldwide by national reference laboratories (Petersen *et al.*, 2002; Anonymous, 2007b; I).

In January 2000, the WHO launched WHO Global Foodborne Infections Network (GFN) (formerly known as Global Salm Surv (GSS)), a global effort to enhance laboratory-based surveillance of *Salmonella* infections and other foodborne diseases, and to promote prevention and control activities. Enhancing worldwide serotyping of *Salmonella* is a key objective of WHO GFN and is facilitated by bench training at international capacity building courses.

To ascertain the performance of participating laboratories, and thereby promote enhanced laboratory-based surveillance, an External Quality Assurance System (EQAS) was established as a part of WHO GFN in 2000 (Petersen *et al.*, 2002; I). Each year, EQAS distributes a set of blinded bacterial cultures for identification, serotyping and antimicrobial susceptibility testing. A key component of this program is the Internet based Country Data Bank (CDB), to which member countries are encouraged to annually upload data on the 15 most common *Salmonella* serovars (<http://www.antimicrobialresistance.dk>).

The results of the WHO GFN EQAS data from 2000 to 2007 revealed that a total of

249 laboratories in 96 countries participated in annual EQAS testing at least once (Figure 1) (I).



*Figure 1. Countries marked in red (dark grey) and yellow (light grey) have participated in at least one EQAS iteration. Countries marked in red both managed the QA threshold of the EQAS and submitted data to the CDB from between one to three years. These data were included the recent CDB study. Countries marked in green (medium grey) did not participate in any of the programmes (I; II).*

During the seven EQAS iterations, a total of 756 reports were received from the participating laboratories. Cumulatively, 76% of participating laboratories submitted data for all eight strains and 82% of the strains were correctly serotyped. The goal of the EQAS program is for all participating laboratories to perform *Salmonella* serotyping with a maximum of one error. The percentage of laboratories reaching the threshold reporting one or zero errors increased significantly ( $p=0.04$ ), from 48% in 2000 to 68% in 2007. In each EQAS iteration, 84% to 96% of the laboratories correctly serotyped the *S. Enteritidis* isolate that was included in the panel of test strains as an internal quality control strain. Regional variations in performance were observed, with laboratories in Central Asia and the Middle East performing less well overall than other regions. Errors that resulted in incorrect serovar identification were usually caused by difficulties in the detection of the phase II flagellar antigen, or differentiation within antigen complexes. Some of these errors likely are related to the quality of the antisera available (I). The same conclusion, that the main problem existed in detecting the H antigens, was reached by the National Institute for Public Health and the Environment (RIVM) which served as the

community reference laboratory for *Salmonella* as designated by the European Commission. They evaluated 26 European national reference laboratories in 2007 and found that 98% and 96% of the laboratories correctly serotyped the isolates using O and H antigen, respectively (Anonymous, 2007b).

In 2008, the global distribution of *Salmonella* serovars per country, based on data from the WHO GFN CDB, were summarised and analysed in search for trends from 2001 to 2007. All data included were based on reliable data from countries which managed the quality assurance threshold of the WHO GFN EQAS (Figure 1) (V).

The data showed that, in all regions with exception of the Oceania and North American, *S. Enteritidis* and *S. Typhimurium* ranked as the first and second most common serovars, respectively. In the North American and the Oceania regions these two serovars ranked in the

opposite order. Globally the overall proportion for both serovars decreased over time with *S. Enteritidis* decreasing from 44.2% to 41.5% and *S. Typhimurium* decreasing from 18.9% to 15.0% (Figure 2) (V). This was mainly due to a

significant decreasing trend ( $p < 0.01$ ) in the proportion of *S. Enteritidis* in developing countries and a non-significant decreasing trend ( $p = 0.16$ ) in the proportion of *S. Typhimurium* in developed countries.

In addition to *S. Enteritidis* and *S. Typhimurium*, *S. Infantis*

was among the serovars

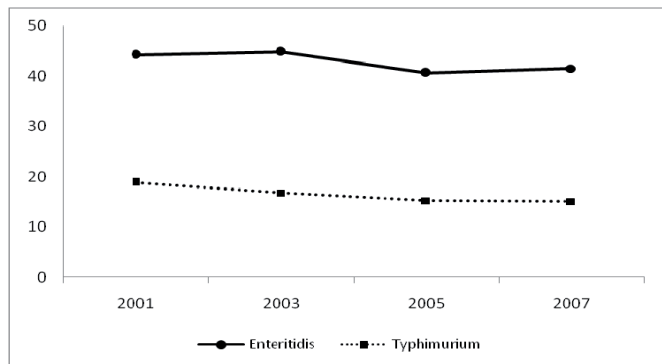


Figure 2. Overall proportion in 37 selected countries over time.(V)

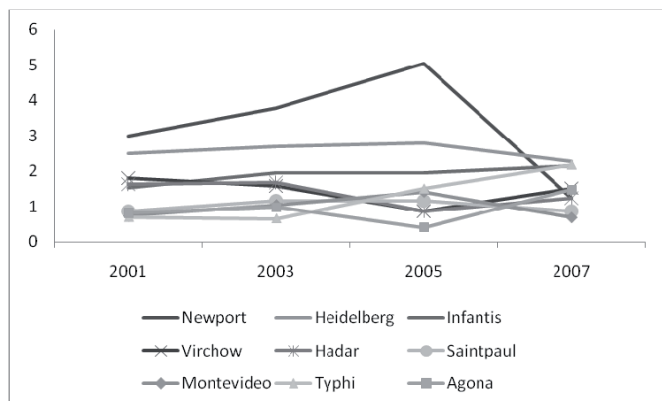


Figure 3. Overall proportion in 37 selected countries over time.(V)

observed in all regions. Globally, the overall proportion of *S. Infantis* increased over the years, from 1.5% to 2.2%. However, no statistically significant increasing trend was detected ( $p=0.76$ ) (Figure 3). *S. Infantis* ranked as the fifth most common serovar in the European region. *S. Agona* was frequently observed in Asia and Latin America, ranking as the third most common serovar. *S. Agona* also ranked as the top seven serovar in Europe and the top 13 in North America. Overall, the proportion of this serovar increased from 0.8% to 1.5% between 2001 and 2007. A slight decrease in the overall proportion over time was seen for *S. Heidelberg*, from 2.5% to 2.3% (Figure 3). This serovar was more common among developed countries. *S. Heidelberg* ranked in top four in North America. However, lower frequencies were seen in Europe (top 9) and Latin America (top 19).

*S. Virchow* was common only in Asia, Europe and the Oceania regions, but with a high proportion. The overall proportion oscillated over the years (Figure 3) and, since 2005, an increase was seen among developed countries while a similar proportionate decrease was reported by developing countries.

High frequencies of *S. Thompson* were seen in Europe and North America. *S. Newport* also was reported as a top serovar by these two regions, in addition to Latin America. Nevertheless, the overall proportion over time of *S. Newport*, which was increasing in the initial years, decreased from 5.0% in 2005 to only 1.2% in 2007 (Figure 3).

*S. Oranienburg* was observed only in North and Latin America, ranked 10<sup>th</sup> and 15<sup>th</sup>, respectively. *S. Hadar* and *S. Montevideo* were reported by almost all regions, however, the frequencies varied considerably. *S. Hadar* ranked 3<sup>rd</sup> in Europe, but lower in the other regions. In general, the overall proportion remained at similar levels over the years, with the exception of a slight decrease in 2005 (Figure 3). Finally, *S. Montevideo* was more common in North and Latin America while *S. Saintpaul* was more predominant in Oceania and North America. These two serovars exhibited similar trends over time, increasing from 2003 to 2005, followed by a decline in 2007 (Figure 3). The survey concluded large differences among the top 20 most commonly isolated serovars between regions but lesser differences between the top 15 most commonly isolated serovars between countries within the same region. Nevertheless, a few serovars are more frequent than others in many of the regions and countries.

Several national surveillance reports and scientific articles support the observations described by Hendriksen *et al.* (II). In Europe, surveillance data from 23 countries between 2006 and 2007

among humans showed that *S. Enteritidis* was ranked first, but decreasing, and that *S. Typhimurium* was fairly consistent over time and ranked second (Anonymous, 2009b). In addition, *S. Infantis* was ranked third followed by *S. Virchow* (top 4), *S. Newport* (top 5), and *S. Hadar* (top 7). However, both *S. Thompson* and *S. Heidelberg* were not listed among top 10 among the 23 countries in Europe (Anonymous, 2009b). In the United Kingdom, data from 1983 to 2007 revealed the same ranking of *S. Enteritidis* and *S. Typhimurium* as for all Europe but with a decreasing tendency for both serovars (Anonymous, 2007f). In contrast, the Danish data from 2007 showed an opposite ranking of *S. Enteritidis* and *S. Typhimurium* (Anonymous, 2009a). In South America, *S. Typhimurium* was ranked first and *S. Enteritidis* second in 2008 (Anonymous, 2008c). In addition, the data also showed that *S. Isangi* was highly frequent and ranked third followed by *S. Dublin* and *S. Virchow* (Anonymous, 2008c). The same tendency of ranking *S. Enteritidis* and *S. Typhimurium* was observed since 1997 in the United States (Olsen *et al.*, 2001; Anonymous, 2008e), in China (Henan province) between 2006 and 2007 (Xia *et al.*, 2009), and Taiwan between 1998 to 2002 (Lauderdale *et al.*, 2006). Also in India, *S. Typhimurium* was ranked before *S. Enteritidis* between 2001 to 2005 (Kumar *et al.*, 2009) and in New Zealand in 2005 to 2008 (Anonymous, 2008a).

In the United States, *S. Newport* was ranked third followed by *S. Heidelberg* (Anonymous, 2008e). The distribution of serovars in Southeast Asia is slightly different from the global trend in general. In the Philippines, Hong Kong, and Sri Lanka, *S. Typhimurium* was ranked before *S. Enteritidis* whereas it was the opposite in Singapore, South Korea, and Thailand (Lee *et al.*, 2009; VI). However, in most of these countries another non-typhoidal *Salmonella* (NTS) ranked either as the top serovar or in between *S. Enteritidis* and *S. Typhimurium* in prevalence. In general, the serovars following *S. Typhimurium* and *S. Enteritidis* were *S. Weltevreden*, *S. Stanley*, *S. Choleraesuis*, *S. London*, *S. Agona*, *S. Rissen*, *S. Anatum*, *S. Panama*, and *S. Virchow* (Lee *et al.*, 2009, VI). In Taiwan, the distribution of serovars revealed large similarities with Southeast Asia. Here, *S. Stanley* was listed as the third most common serovar followed by *S. Schwarzengrund*, *S. Newport*, *S. Albany*, *S. Virchow*, *S. Weltevreden*, and *S. Agona* (Lauderdale *et al.*, 2006). China (Henan province) did not share the same serovars as Taiwan. In Henan province, *S. Derby* ranked third followed by *S. Indiana*, *S. Litchfield*, *S. Thompson*, and *S. Agona* (Xia *et al.*, 2009).

The complexity of the global distribution of *Salmonella* serovars in humans is enormous as it is influenced by multiple factors such as animal and environmental reservoirs and complex route of

transmission. It will be important for the future to extend the global understanding of the epidemiology of *Salmonella* in not only humans but also in all animal reservoirs.

## HUMAN EPIDEMIOLOGY

### *The public health burden of Salmonella infections.*

Food-borne diseases have been estimated to infect up to 76 million people in the United States annually. This would equal one fourth of the people infected in the developed world per year if these data were extrapolated. The burden of salmonellosis is expected to be much greater in the developing parts of the world (Schludt *et al.*, 2004). *Salmonella* is overall the most common food-borne pathogen in the United States, however, in some states *Campylobacter* is more prevalent than *Salmonella* (Anonymous, 2009c). In Europe, *Campylobacter* are more frequent than *Salmonella* (Anonymous, 2008d). According to the WHO, humans NTS infections constitute a major public health burden on society and represent a huge cost for many countries ([www.who.int](http://www.who.int)). In 2000, it was estimated that in the United States, NTS resulted in 1.4 million infections annually in a population of about 293 million inhabitants with approximately 168.000 visits to the general practitioner (GP). A total of 16.430 people were hospitalized resulting in 582 deaths (Mead *et al.*, 1999; McDermott *et al.*, 2006). A similar study was conducted the same year in the United Kingdom. This revealed that 41.616 NTS cases occurred each year with 15.036 laboratory confirmations among a population of 60 million people, resulting in 1.516 hospitalization and 119 deaths (Adak *et al.*, 2002; McDermott *et al.*, 2006). This was approximately twice as many hospitalizations and deaths in the United States compared to the United Kingdom.

Recently, the financial burden of NTS was estimated in the United States based on FoodNet data. The data revealed that costs of medical care, lost productivity, and mortality exceeded more than \$ 3.6 billion annually (Voetsch *et al.*, 2004; McDermott *et al.*, 2006). In comparison, the annually costs of NTS in Denmark are estimated to be \$ 15.5 million which was approximately four times less than in the United States ([www.who.int](http://www.who.int)).

A few countries around the world have an established laboratory-based surveillance of NTS, or have recently improved the data quality and reporting to implement programmes measuring the burden of salmonellosis. One of the measurements in the surveillance reports are the notification, incidence, or isolation rate per 100.000 inhabitants all estimating the burden on the society



caused by salmonellosis (Figure 4) (Bangtrakulnonth and Tishyadhigame; 2006; Aissa *et al.*, 2007; Anonymous, 2008c; Anonymous, 2008d; Anonymous, 2008e).

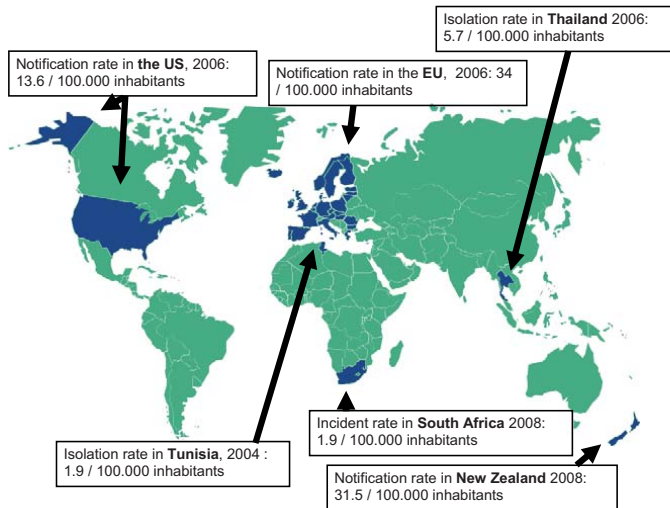


Figure 4. Examples of incident, notification, and isolation rate in different parts of the world. (Bangtrakulnonth and Tishyadhigame; 2006; Aissa *et al.*, 2007; Anonymous, 2008c; Anonymous, 2008d; Anonymous, 2008e).

In the United States, the National *Salmonella* Surveillance System reported in 2006 an increase of 12.3% of NTS compared with 2005 due to increased reporting. In general, the level decreased compared with data from 1996 (Anonymous, 2008e). The number of human *Salmonella* cases also seems to have declined in Tunisia based on data collected from 1994 to 2004 with the lowest isolation rate monitored in 2004 for the entire surveillance period (Aissa *et al.*, 2007). The 2008 Annual Surveillance Report from New Zealand showed, as many other country reports, a decreasing notification rate of NTS (Anonymous, 2008a). Based on data from 29 countries, the number of salmonellosis cases in Europe decreased by 8% between 2005 and 2006 (Anonymous, 2008d). In 2006, the National *Salmonella* and *Shigella* Center in Thailand found 3.758 NTS cases, representing a decrease compared with previously published data showing the number of human cases from 1993 to 2002 (Bangtrakulnonth *et al.*, 2004).

The worldwide reported trend of decreasing human cases caused by NTS seems to be in agreement with what have been observed elsewhere. A decreasing number of *Salmonella* isolates were serotyped from 2001 to 2007 when assessing the global distribution of *Salmonella* serovars in 37 countries worldwide. The data were based on quality data submitted to CDB of the WHO

GFN **(II)**. Despite the decreasing occurrence of NTS infections in human, the problem is still large and is largely preventable and therefore an unnecessary burden on public health.

### *Symptoms and human infections*

The symptoms of *Salmonella* infections usually appear 12 to 72 hours after ingestions of the organism, and include diarrhea, fever, abdominal cramps, nausea, and sometimes vomiting but asymptomatic infections may also occur. The illness usually lasts from 4 to 7 days but are in most cases self limiting ([www.cdc.gov](http://www.cdc.gov); [www.who.int](http://www.who.int); McDermott *et al.*, 2006; Anonymous, 2007f). NTS gastroenteritis will develop into bacteremia in about 5% of cases. Bacteremia often requires hospitalization with a prolonged course of illness and could potentially result in a fatal outcome (Hohmann *et al.*, 2001; Jones *et al.*, 2008). A cohort study with almost 49.000 participants showed that people with gastrointestinal infections caused by NTS have an excess mortality with a relative risk of 13.31 up to a month after being infected (Helms *et al.*, 2003). Several studies have described certain serovars such as *S. Dublin* and *S. Choleraesuis* often being associated with invasiveness (Hohmann *et al.*, 2001; Helms *et al.*, 2002; Chiu *et al.*, 2004; Jones *et al.*, 2008; **V**; **VI**).

An observational study based on patient data from 11.656 isolates (2002 – 2007) estimated the risk factors of the ten most common *Salmonella* serovars from Thai patients. The data showed that 87.4% of 681 *S. Choleraesuis* isolates originated from blood samples with a significant increased odds ratio of 44.00 (95% CI 34.3 – 56.5) when compared to other NTS serovars. *S. Enteritidis*, *S. I* [1],4,[5],12:i:-, *S. Typhimurium*, and *S. Panama* did also seem to be highly invasive when compared to other NTS serovars (**VI**). These data correspond well with an investigation describing the differences in the outcome of salmonellosis based on the various serovars (Jones *et al.*, 2008). Sixty percent and 67% of all *S. Choleraesuis* and *S. Dublin* infections, respectively, require hospitalization compared with other serovars. However, *S. Heidelberg*, *S. Poona*, *S. Panama*, *S. Virchow*, *S. Paratyphi B* var. *Java* and *S. Sandiego* also seemed to more frequently cause invasive diseases (Jones *et al.*, 2008).

Several studies have shown that invasive NST is endemic in sub-Saharan Africa (Morpeth *et al.*, 2009; Vandenberg *et al.*, 2009). In some of those countries the mortality in children caused by NST bacteremia exceeds the burden of childhood malaria (Morpeth *et al.*, 2009). In the Democratic Republic of Congo, a retrospective study from 2002 to 2006 in one hospital showed

that 59% of all bloodstream infections in children were caused by NTS. The data revealed that *S. Enteritidis* and *S. Typhimurium* were responsible for up to 82.8% of the cases (Vandenberg *et al.*, 2009). The study highlighted that many NTS invasive infections were nosocomial and resulted in prolonged hospitalization, posing a significant problem in developing countries. Little is known about human NTS infections in Africa and many other developing countries, but the limited data have shown that NTS infection often are associated with invasiveness and severe outcome. There is an urgent need to address this problem in order to elucidate the mechanisms responsible for the severe outcomes.

#### *Age, season, and risk factors*

Human *Salmonella* infections are age specific and affect mostly children, elderly people and immunological compromised patients (Hohmann *et al.*, 2001; Anonymous, 2008c; Anonymous, 2008d; Anonymous, 2008e; Jafari *et al.*, 2009; **VI**).

The reason for the typical age pattern is believed to be a result of children acquiring immunity to *Salmonella*, and deteriorating

immune status in the elderly. This observation was supported by

Hendriksen *et al.* who showed that from 2002 to 2007 in Thailand,

32.6% of all *Salmonella* cases were observed among children from 0 to 5

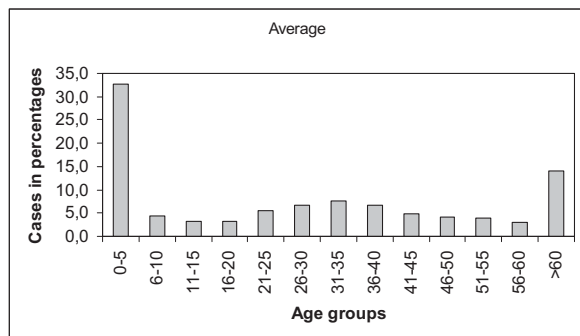


Figure 5. Distribution of age among Thai patients with salmonellosis from 2002 -2007 (**VI**)

years of age and peaked again with

14.0% of all cases in people older

than 60 years in Thailand (Figure 5) (**VI**). The previously mentioned risk factor analysis from

Thailand showing an odds ratio between 1.63 (95% CI 1.1-2.5) and 1.51 (95% CI 1.1-2.0) in the age group of 6-20 and 21-40 years for being infected with *S. Choleraesuis* compared to other

NTS infections (**VI**). In the risk factor analysis, additional serovars seemed to be age specific

such as *S. Anatum*, *S. Enteritidis*, and *S. Weltevreden*, which mainly affected people older than 6

years. In contrast, *S. Stanley*, *S. Panama*, and *S. I* [1],4,[5],12:i:- predominately infected children less than 6 years of age (**VI**).

Several surveillance reports have illustrated the general infection pattern of human salmonellosis associated with yearly seasonality, with the summer months being the season having the highest incidence of infections (Anonymous, 2008a; Anonymous, 2008c; Anonymous, 2008d; Cho *et al.*, 2008; **VI**). In Tunisia, however, this general pattern of infection seemed not to be consistent with the hypothesis that most infections occur in the summer months. *S. Enteritidis* infections peaked in January followed by *S. Livingstone* peaking in April, whereas *S. Corvallis* peaked in October (Ben-Aissa *et al.*, 2007). Ben-Aissa *et al.* did not indicate a reason for the skewed seasonal

pattern but one possibility could be that this was the same time of the year when the serovar peaked in the animal reservoir. In Thailand, the seasonality of *Salmonella* infections were in general in agreement with other studies having most infections in the summer period (Figure 6) (**VI**). Nevertheless, Hendriksen *et al.* describe that infections caused by

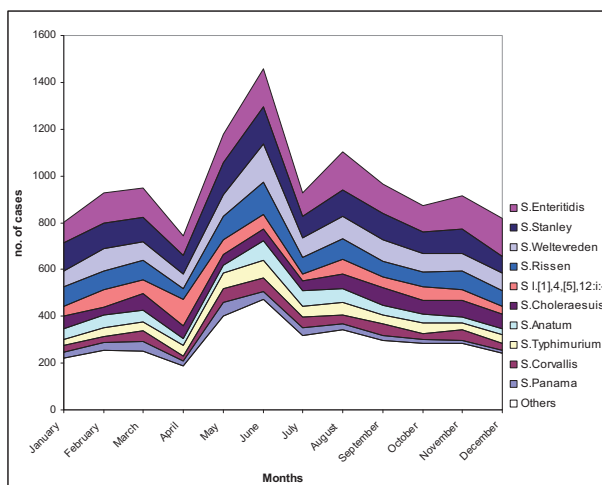


Figure 6. Seasonal variation of *Salmonella* cases from Thai Patients between 2002 - 2007. (**VI**)

*S. Enteritidis* had the highest odds ratio of 1.2 (95% CI 1.0-

1.5) during the winter time. For infections caused by *S. I* [1],4,[5],12:i:- and *S. Panama*, however, spring time seemed to constitute the highest risk with an odds ratio of 1.8 (95% CI 1.5-2.3) and 1.5 (95% CI 1.2-2.0). *S. Choleraesuis* was observed to pose the highest risk with an odd ration of 1.4 (95% CI 1.1-1.9) during autumn (**VI**) which was supported by another study (**V**).

The use of available surveillance data for descriptive analysis and source attribution, or even more advanced analysis, should be of a high priority of all countries in order to facilitate a more direct and targeted effort to minimize the burden of salmonellosis in high risk populations.

#### *Antimicrobial treatment and antimicrobial resistance*

Antimicrobial treatment is not routinely recommended for empiric treatment of gastrointestinal infections caused by NTS in healthy people as the infection often is self limiting. Antimicrobial

treatment should be given to patients with severe illness, immunosuppression or patients suffering from bacteraemia (Hohmann *et al.*, 2001). Treatment with first line antimicrobials should include ampicillin, chloramphenicol or trimethoprim + sulfamethoxazole (Hohmann *et al.*, 2001; McDermott *et al.*, 2006). The choice differs by region and chloramphenicol is not used in most developed countries, but is common in developing countries. Ampicillin and trimethoprim + sulfamethoxazole are good choices, but many do not even considering them and choose instead fluoroquinolone or 3<sup>rd</sup> generation cephalosporins.

Unfortunately, the recent increased development of resistance to many antimicrobials often leaves the GP with no alternative than to treat the infection with either a fluoroquinolone or 3<sup>rd</sup> generation cephalosporins. These antimicrobials are routinely used for empiric treatment if the susceptibility of the isolates is unknown or if the patient suffers from bacteremia (Hohmann *et al.*, 2001). For paediatric patients, treatment with a fluoroquinolone is contraindicated, and practitioners will rely on ceftriaxone or another 3<sup>rd</sup> generation cephalosporin (Hohmann *et al.*, 2001).

Several studies from the United States, Canada and Denmark have shown an increased risk of hospitalization or even death associated with multi-drug resistant NTS compared with pansusceptible NTS. (Holmberg *et al.*, 1987; Lee *et al.*, 1994; Mølbak *et al.*, 1999; Helms *et al.*, 2002; Martin *et al.*, 2004; Helms *et al.*, 2004; Varma *et al.*, 2005a; Varma *et al.*, 2005b). In a Danish study, an increased risk of invasive illness has been observed with 3.5% of the patients investigated being hospitalized. An increased mortality was recorded in 1.2% of the patients in up to two years after the infection. In both cases the infections were associated with quinolone or multi-drug resistant *S. Typhimurium* (Helms *et al.*, 2002; Helms *et al.*, 2004).

Recently, multi-drug resistant NTS have increased and have reached an alarming level worldwide. While the extent varies, the increased level of multi-drug resistant NTS has become a problem in all countries. Data have revealed that countries in Southeast Asia and Africa tend to have a high level of resistant NTS (Collard *et al.*, 2007; Lee *et al.*, 2009; Vanderberg *et al.*, 2009). Several publications have described the increasing occurrence of multi-drug resistant NTS and isolates resistant to both fluoroquinolone and 3<sup>rd</sup> generation cephalosporins in Southeast Asia and Africa (Archambault *et al.*, 2006; Lauderdale *et al.*, 2006; Aarestrup *et al.*, 2007; Collard *et al.*, 2007; Vandenberg *et al.*, 2009; Lee *et al.*, 2009; **III**; **IV**; **V**). Lee *et al.* recently described the level of antimicrobial resistance from 2003 to 2005 in seven Southeast Asia countries. They

found that Taiwan and Thailand demonstrated an alarming high frequency of resistance to fluoroquinolones and 3<sup>rd</sup> generation cephalosporins. These findings were also supported by Sirichote *et al.* who found cephalosporinases producing *S. Choleraesuis* from Thai patients and a Danish traveler to Thailand harbouring both *bla*<sub>CTX-M-14</sub> gene and *bla*<sub>CMY-2</sub> gene (V). Hendriksen *et al.* showed that among 33 Thai patients infected with *S. Rissen* 36%, 27%, 33%, 30%, 27%, and 88% of the isolates were resistant to ampicillin, chloramphenicol, spectinomycin, streptomycin, sulfamethoxazole, trimethoprim, and tetracycline, respectively (III). Another study highlights the same worrisome frequency of multi-drug resistance in children from Ethiopia (IV). The investigation revealed that among 43 *S. Concord* isolates, all isolates were resistant to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and trimethoprim. In addition, 97%, 97%, 69%, and 14% of the isolates showed resistant or decreased susceptibility to ceftriaxone, gentamicin, tetracycline, and ciprofloxacin, respectively. All of the isolates resistant to ceftriaxone harboured the *bla*<sub>CTX-M-15</sub> gene and 13 of the isolates also the *bla*<sub>SHV-12</sub> gene (IV). In response to worldwide increases in multi-drug resistance among human bacterial pathogens, the WHO has developed a list ranking the critically important antimicrobials. This categorization of antimicrobials is prioritized according to their importance in human medicine, and is intended to help assess the risks associated with resistance (Anonymous, 2007e; Collignon *et al.*, 2009). In addition, individual countries without a strict antimicrobial policy should consider lowering the consumption of antimicrobials, ban antimicrobial growth promoters and enforce prescription-only policies to accommodate the increasing frequency of multidrug resistant pathogens worldwide.

## MAIN RESERVOIRS

As a zoonotic foodborne bacterium, *Salmonella* has reservoirs in various animals. The most common domesticated animal hosts are chickens, pigs, and cattle; but many other domestic animals as well as a wide range of wild animals can also harbour this organism. Because of the ability of *Salmonella* to contaminate meat during slaughter and to survive in fresh meats and meat products that are not thoroughly heated, animal products constitute a main vehicle of transmission. Another important vehicle of transmission is eggs that are contaminated on the surface or in the interior of the egg. Finally, produce and other vegetables that are contaminated

with animal manure during growing or processing are increasingly recognized as an important source of human *Salmonella* infections

#### *Host adapted and host restricted serovars*

NTS have a wide range of hosts and reservoirs which mostly have been associated with agricultural product. Some of the NTS are host adapted or host restricted while others are non-specific and cause infections in various hosts, leading to their division into two separate groups (Uzzau *et al.*, 2000; Cray *et al.*, 2000).

The host restricted serovars cause disease in a limited number of animal species such as *S. Abortusequi* (horses), *S. Gallinarum* (poultry), *S. Pullorum* (poultry), *S. Typhimurium* (swine), and *S. Abortusovis* (sheep). The host adapted serovars are most prevalent in one animal species, but are also able to cause severe illness in a limited number of other hosts. These serovars include *S. Choleraesuis* (predominantly in swine and human) and *S. Dublin* (predominantly in cattle and human) (Uzzau *et al.*, 2000; Chiu *et al.*, 2004). There are limited data describing the reasons these serovars only affect a limited number of hosts compared to non-specific serovars which colonize a broad range of animals and humans (Uzzau *et al.*, 2000).

Some decades ago, *S. Choleraesuis* was in many countries one of the most predominant serovars isolated from swine (Cray *et al.*, 2000). Recently, the prevalence decreased in Europe and is not presently listed among the top serovars isolated from swine (Uzzau *et al.*, 2000; Anonymous, 2007f; Anonymous, 2009a; Anonymous, 2009b). In many countries, *S. Choleraesuis* is now believed to be eradicated. However, the incidence in the United States does not seem to have followed the same path as in Europe and *S. Choleraesuis* have not decreased to a similar level. Thus, *S. Choleraesuis* represent a major swine pathogen in the United States and costs the producers an estimated \$100 million annually (Gray *et al.*, 1996; Uzzau *et al.*, 2000). Despite the high prevalence in animals, it rarely causes human illness in the United States, with approximately 40 cases annually (Foley *et al.*, 2008). Today, *S. Choleraesuis* is mainly a problem in Southeast Asia, especially in Taiwan and Thailand, with high frequencies of human illness. This may be due to little effort to prevent and control this serovar, but also as a consequence of local small scale farming where infection control is difficult (Chiu *et al.*, 2004; Foley *et al.*, 2008; Lee *et al.*, 2009; V; VI).

In many European countries, surveillance shows that *S. Dublin* is the most commonly isolated serovar from bovine meat, exceeding the level of *S. Typhimurium* (Anonymous, 2009b). In 2007, it was the most common serovar in bovine meat from both Denmark and the Netherlands with 68.2% and 63.3% of isolates, respectively. In the same year, *S. Dublin* was also ranked as the most common serovar isolated from cattle herds in Austria (33.3%), Belgium (66.3%), Denmark (52.3%), Ireland (82.5%), the Netherlands (63.6%), Sweden (34.8%), and the United Kingdom (65.1%) among 15 European countries including Norway (Anonymous, 2009b).

It is difficult to estimate the frequency of *S. Dublin* among cattle isolates from outside of Europe due to limited data. *S. Dublin* is not listed among the most common serovars in cattle from either the United States, Canada or New Zealand (Wray *et al.*, 2000; Anonymous, 2007c; Anonymous, 2007d; Anonymous, 2008b).

Because the reservoirs of the host adapted serovars are known, and limited in number, countries with a high number of human infections caused by these serovars could implement control strategies to eradicate the serovars among the reservoir in order to limit the transmission to man.

#### *Host non-specific serovars*

The non-specific serovars are not restricted to a single host but able to colonize, and on occasion, cause severe illness or gastroenteritis in a wide range of animal species (Uzzau *et al.*, 2000).

Today, more than 2,579 different serovars are known to man (Grimont *et al.*, 2007) but only a limited number of approximately 50 serovars are predominantly found in domestic animals. The primary reservoirs for the majority of the remaining serovars remain obscure.

Several factors complicate a clear picture of the true link between the serovars and the animal reservoir such as the production systems (intensive / free range), irrigation (manure) and contamination of food sources (cross contamination). In addition, only a limited number of countries have established a systematic integrated laboratory-based surveillance system, which includes data from both food and animals. Despite these factors, some serovars appear to be more frequently associated with certain animal species and / or production systems than others.

In Figure 7, the most commonly isolated serovars in 2007 from pig meat in eight European countries are illustrated (Anonymous, 2009b). Only two serovars; *S. Typhimurium* and *S. Derby* were common for all nine countries. The same distribution of serovars was observed for pig herds for 17 European countries. A comparison with the incidents data from the United Kingdom



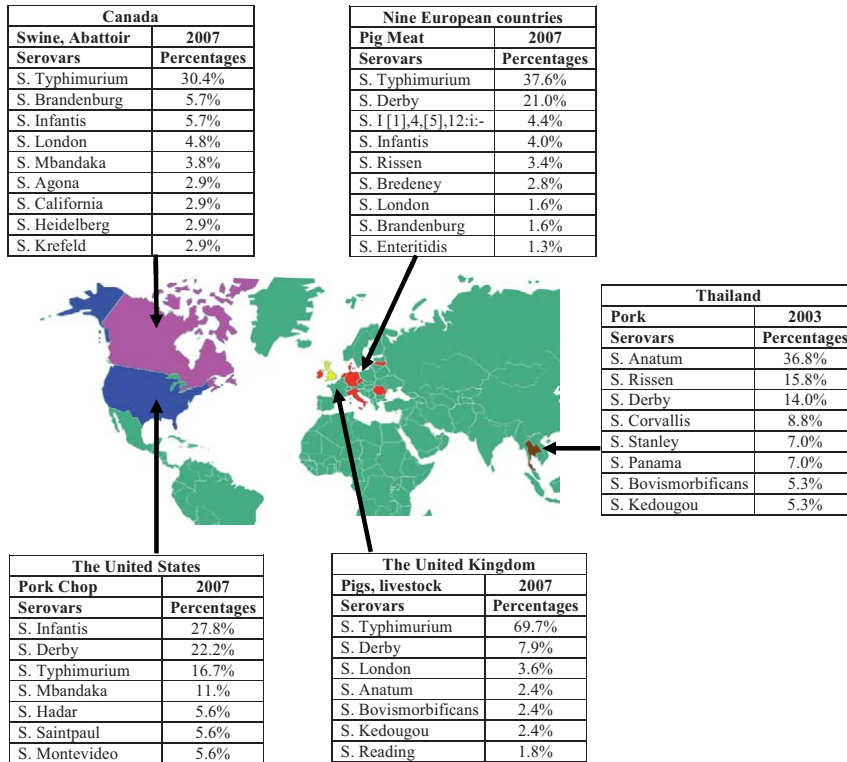


Figure 7. Rank of the most common serovars associated with pigs or pork in different parts of the world. (Anonymous, 2007c; Anonymous, 2007f; Vindigni *et al.*, 2007; Anonymous, 2008b; Anonymous, 2009b).

reveals the same complexity in pigs (livestock). In 2007, only three of the serovars described in the overall prevalence of pig meat from the nine European countries were listed among the top seven serovars isolated from pigs in the United Kingdom (Anonymous, 2007f). These data revealed that even within small geographical area huge changes may be present. Differences were also revealed when compared with 2007 data from the United States and Canada (Anonymous, 2008b; Anonymous, 2007c; Anonymous, 2007d). The top three most common serovars from pork chops in the United States were the same as observed in pig meat from Europe (Anonymous, 2007c). However, the list revealed additional serovars when surveyed marked hogs; *S. Johannesburg* (9.9%), *S. Saintpaul* (6.4%), *S. Adelaide* (4.9%), *S. Agona* (4.4%), and *S. Hadar* (3.9%) (Anonymous, 2007c). The list was further expanded when assessing the data from Canada from swine abattoirs (Anonymous, 2008b). In Thailand, a different set of serovars were reported from pork isolated in 2004 (Figure 7) (Vindigni *et al.*, 2007). It is still unknown if

the huge number of serovars isolated from swine is a result of a better surveillance or if other factors contribute to the increase of serovars (Lee *et al.*, 2009).

It appears as the number of different serovars associated with cattle are just as diverse as for swine. In the European surveillance of bovine meat in 2007, three of the five countries have reported only two to three serovars (Figure 8) (Anonymous, 2009b).

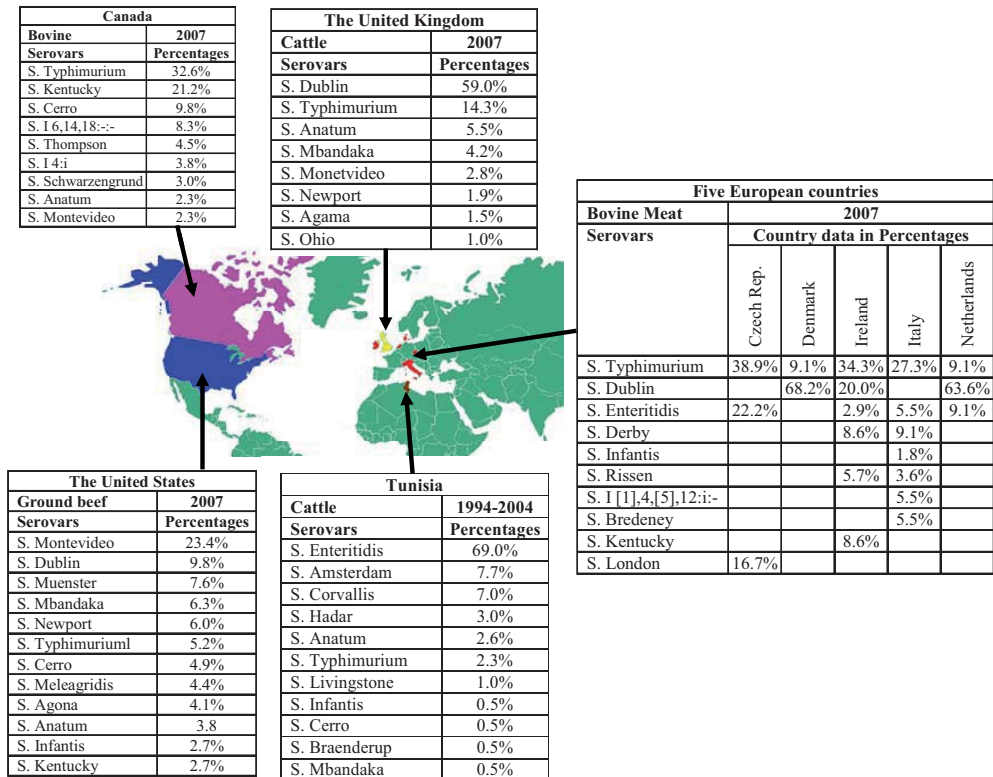


Figure 8. Rank of the most common serovars associated with cattle or bovine meat in different parts of the world. (Ben-Aissa *et al.*, 2007; Anonymous, 2007d; Anonymous, 2007f; Anonymous, 2008b; Anonymous, 2009b).

In the Netherlands and Denmark, *S. Dublin* was the most prevalent serovar in bovine meat.

However, in Ireland and Italy numerous serovars were isolated from bovine meat thus in both countries *S. Typhimurium* was the most frequent serovar observed. The distribution of serovars in cattle herds from 15 European countries including Norway is quite different from the serovars in bovine meat from the five European countries. The only serovars ranked similarly in cattle herds compared to bovine meat are *S. Typhimurium* and *S. Dublin* whereas several serovars, such

as *S. Anatum*, *S. Havana*, *S. Goldcoast*, *S. Give*, *S. Bovismorbificans*, are not listed among the 10 most frequently isolated serovars in bovine meat (Anonymous, 2009b). The distribution of serovars in cattle based on livestock incidents data was for the United Kingdom different from the overall European data in 2007 (Anonymous, 2007f). In Tunisia, the prevalence of serovars in cattle was quite different from Europe. Between 1994 to 2004, *S. Enteritidis* was ranked as the most common followed by *S. Amsterdam* and *S. Corvallis* (Ben-Aissa *et al.*, 2007). In the United States, *S. Montevideo* was the most common isolated serovar among ground beef followed by *S. Dublin* (Anonymous, 2007d). *S. Cerro* seemed to be frequent in Canada where it was ranked as the third most common serovar in cattle (Anonymous, 2008b).

*S. Enteritidis* is probably best known for its association with poultry (*Gallus gallus*) and egg. Today, *S. Enteritidis* no longer ranks among the most common serovars in chickens in many countries and the prevalence is decreasing in both egg and chicken. In 2007, *S. Kentucky* was listed as the most common serovar in broiler meat from Europe. However, this included only four (Austria, Czech Republic, Ireland, and Slovakia) out of 11 countries. All of the 11 countries in the European survey have ranked *S. Enteritidis* second (Figure 9) (Anonymous, 2009b). In 2007, *S. Enteritidis* is still ranked as the most common serovar isolated from flocks of *Gallus gallus* among 14 European countries with exception of Germany where *S. Livingstone* is the most predominant serovar. Interestingly, a comparison of predominant serovars between chicken meat (*Gallus gallus*) and flocks of *Gallus gallus* among European countries reveal some differences in the ranking of serovars shared by both reservoirs. In addition, frequently isolated serovars present in chicken meat such as *S. Kentucky*, *S. Agona*, *S. Ohio*, and *S. Indiana* are not ranked among the 10 most common serovars in flocks of *Gallus gallus* whereas the opposite is the case with *S. Livingstone*, *S. Mbandaka*, *S. Seftenberg*, and *S. Bredeney* (Anonymous, 2009b). In the United Kingdom, additional serovars were observed being highly frequent among chickens in 2007 (Anonymous, 2007f). The same tendency of *S. Enteritidis* decreasing was observed in 2007 among broilers in the United States (Anonymous, 2007d). The exact same pattern as observed in the United States was seen in Canada where the same two serovars were listed first and second with approximately the same percentages (Figure 9) (Anonymous, 2008b). There is a need to regularly survey the primary animal reservoirs in order to detect the emergence of new and emerging serovars or sub-types such as for instance *S. Typhimurium* DT 104, to elucidate their epidemiology, and epidemic potential, and to conduct targeted interventions to avoid

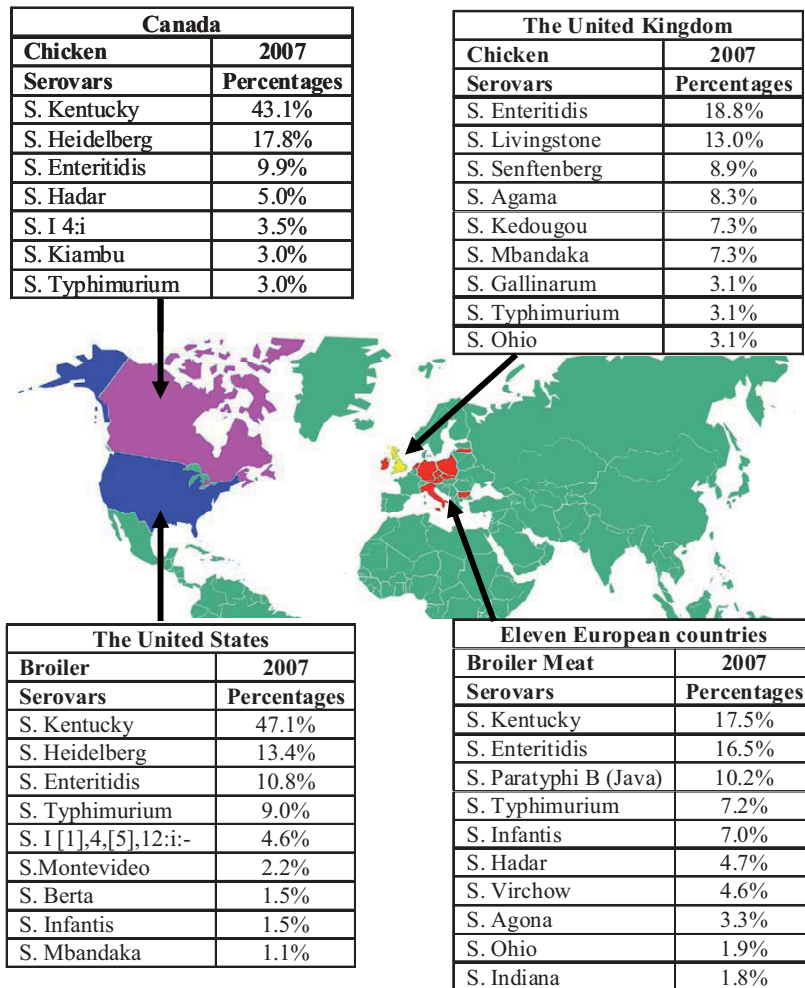


Figure 9. Rank of the most common serovars associated with chicken or broiler meat in different parts of the world. (Anonymous, 2007d; Anonymous, 2007f; Anonymous, 2008b; Anonymous, 2009b).

transmission to humans if necessary. In some countries, the primary animal reservoir might not be cattle, swine or poultry but reptiles or seafood. Several studies have shown that reptiles are reservoirs for *Salmonella* that are able to affect humans (Cooke *et al.*, 2009; Pedersen *et al.*, 2009). Similarly, rare serovars have been suggested to be associated with seafood (Aarestrup *et al.*, 2003) and other serovars have found niches in small production settlements (Raufu *et al.*, 2009).

## TRANSMISSION – LOCAL AND GLOBAL

### *Primary routes of transmission*

The primary reservoirs of NTS are the intestinal tracts of colonised food- and wild animals. In most industrialised countries, food of animal origin is the primary vehicle for human salmonellosis, however multiple routes of transmission has been documented including vector- and waterborne, animal-, human- and environmental contact, as well as many others. In the Netherlands, *S. Typhimurium* isolated from a pig, a calf, and a child on a farm were identical, indicating animal-to-animal and animal-to-human transmission (Hendriksen *et al.*, 2004). *Salmonella* is passed on from the intestinal tract of the host to meat products during slaughter, where faecal contamination often occurs. Human infections are acquired from contaminated meats due to inadequate cooking or poor kitchen hygiene, the latter of which can result in cross-contamination of uncooked foods such as vegetables (Figure 10).

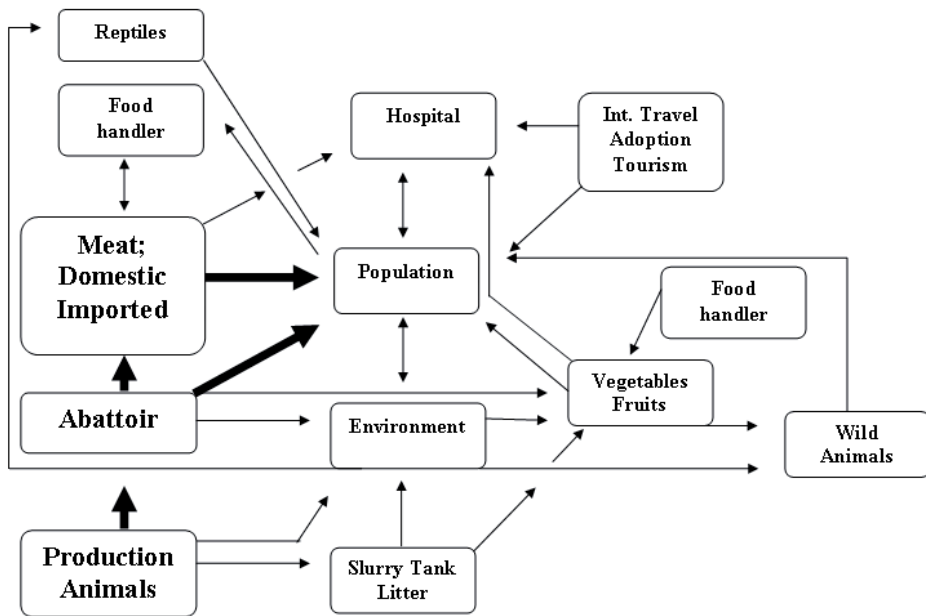


Figure 10. The transmission of *Salmonella* between reservoirs. The thickness of the arrows indicate the most important routes.

In 2007, 26.3% of human infections in Denmark were associated with domestically produced meat products, 11.7% were associated with imported meat, and 48.2% were associated with international travel (Anonymous, 2009a). Waste/manure from the production of food animals or from abattoirs often contains *Salmonella*, which can survive for years outside its host in the environment, sewage, or slurry tanks. Faecally contaminated water from these reservoirs is a major route of *Salmonella* transmission to vegetables and fruits, and can result in human infections. The increased demand from consumers for fresh vegetables and fruits year round has also resulted in an increased level of NTS outbreaks caused by these commodities. Several environmental factors contribute to produce contamination



Figure 11. Integrated production of poultry and fish in Thailand. Courtesy of Andreas Pedersen

by NTS, such as irrigation and rinse of the crops in polluted fresh and waste water, some of which originated from animal production systems (Figure 11) (Sivapalasingam *et al.*, 2003; Hanning *et al.*, 2009). A recent review highlights examples of human outbreaks in the United States from 1950 to 2007 associated with vegetables and fruits. At least 25 different NTS in at least 15 different vegetable or fruit products were responsible for the outbreaks in this time period (Hanning *et al.*, 2009). In 2004, an international outbreak associated with imported Italian rucola lettuce occurred in Sweden, Norway, and the United Kingdom (Nygård *et al.*, 2008). This outbreak was caused by *S. Thompson* and resulted in 21 reported cases, but was believed to be much larger in magnitude. In 2008, a multi-state outbreak in the United States was caused by *S. Saintpaul*. A total of 1,442 persons from 43 states in the United States and Canada were infected, 286 of which were hospitalized and two died. The responsible vehicle was jalapeño peppers imported from Mexico, but Serrano peppers and tomatoes also were believed to have contributed to the outbreak. All of these products were contaminated with *Salmonella* from irrigation water polluted by an animal source (Anonymous, 2008f). A large

number of *Salmonella* infections caused by contaminated vegetables or inadequate cooking could probably be prevented if consumers followed basic kitchen hygiene regimes, such as frequent hand washing, thorough cleaning of raw vegetables, keeping hot items hot and cold items cold, and by the separation of raw meat and vegetables on the cutting board.

### Source attribution

Recently, several countries have initiated the development of systems to attribute sporadic cases of human salmonellosis to specific sources, in the quest to control and prevent salmonellosis.

In Denmark, a source attribution model was applied to retrospectively assess sources of salmonellosis between

1988 and 2004. In 2000 to 2001, 53.1% of all cases were linked to domestically produced food products – mainly table eggs (37.6%). However, the data also showed that 19% of all cases were travel

related and 9.5% were associated with

imported food products – dominated by chickens (Hald *et al.*, 2007). In 2007, the travel associated cases increased considerably to 46% of all cases compared to the 2001 data, due in part to a substantial improvement in reporting travel information, and in part to a reduction in the number of domestically acquired infections (Anonymous, 2009a). Consequently, the infections acquired from domestically produced food items decreased to 19% in 2007. Similarly, a reduction in *Salmonella* cases that could be attributed to imported food products was observed in the 2007 data due to a huge decrease in the number of cases associated to imported chickens, probably due to enhanced control of imported food products. The overall results of the Danish source attribution system revealed that travel associated sources and imported food products were far more important risk factors of human *Salmonella* infections than was consumption of

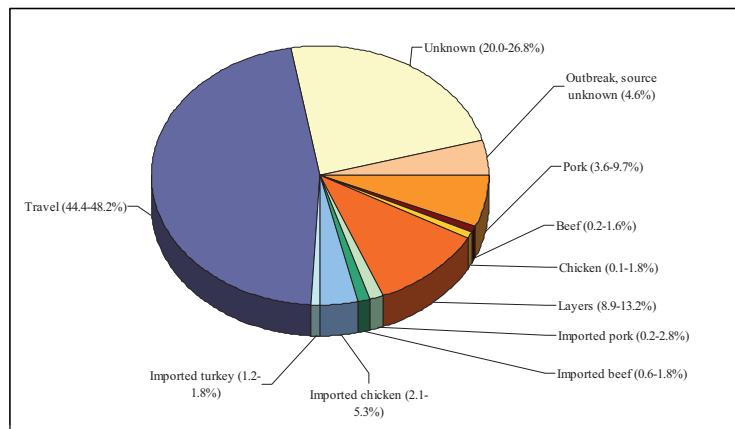


Figure 13. Estimated sources of 1,647 registered cases of human salmonellosis in Denmark, 2007. Source: Danish Zoonosis Centre, National Food Institute, Technical University of Denmark.

domestically produced products in 2007 (Figure 13) (Anonymous, 2009a). Countries with integrated laboratory-based surveillance systems could benefit from attributing the human infections to specific food categories, and other sources, and by this approach, gain knowledge that can enable them to target control, intervention, and prevention strategies to the most important sources.

### *International trade of food and animals*

In the last decade, the international trade of food has increased resulting in increasing import of cheaper food products from countries with little or no control programmes of foodborne pathogens. In addition, many of the imported food products contain multi-drug resistant foodborne pathogens (Aarestrup *et al.*, 2007). Today, international trade of food products contaminated with not only NTS but all foodborne pathogens poses a threat to the public health. A retrospective study investigated the association between imported pig and pork from Germany and Spain and Thai food products and Thai patients with Danish patients all containing or infected with *S. Rissen* (I). The data revealed that six out of nine isolates from imported pork had

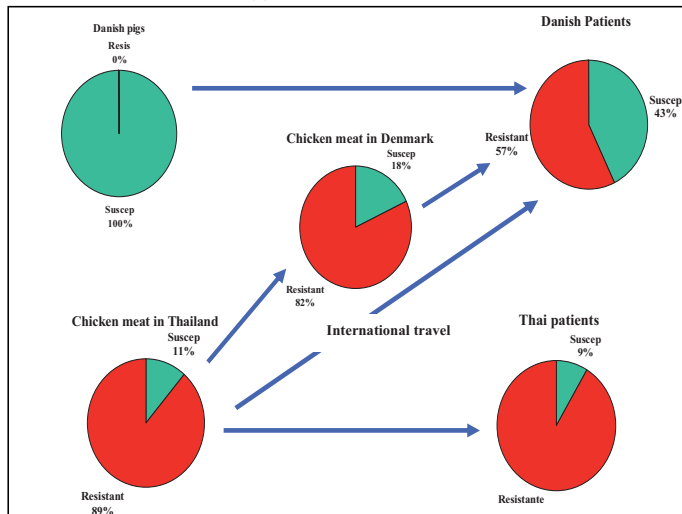


Figure 12. Spread of multidrug-resistant *S. Schwarzengrund* from chickens to humans in Thailand, and from imported Thai food products to humans in Denmark and the United States. Courtesy of Frank M. Aarestrup.

the same genetic Pulsed Field Gel Electrophoresis (PFGE) pattern as a Danish patient. Aarestrup *et al.* described the spread of *S. Schwarzengrund* resistant to nalidixic acid from imported Thai chicken to Denmark (Figure 12). The data showed that *S. Schwarzengrund* from

Danish pigs were susceptible to nalidixic acid, in contrast to isolates from

the Thai chicken. However, Danish patients were infected with both nalidixic acid resistant and susceptible *S. Schwarzengrund* isolates, suggesting that Danish patients were affected by



consumption of Thai chickens imported to Denmark or consumed in Thailand, and domestically produced pigs (Aarestrup *et al.*, 2007).

The import of live animals is a means by which NTS can disseminate between countries. In 2003, the first case of NTS resistant to extended spectrum cephalosporins (ESC) occurred in a pig intended for breeding, which was imported into Denmark from Canada (Aarestrup *et al.*, 2004). Microbiological study of the intestinal contents of this animal revealed the presence of a *S. Heidelberg* strain harboring the *bla*<sub>CMY-2</sub> gene. While rare or absent in most regions of the globe, *S. Heidelberg* ranks amongst the most prevalent causes of human salmonellosis in Canada and the United States. In addition, an increase in ESC resistance has been observed both by the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) (Andrysiak *et al.*, 2008) and the U.S. National Antimicrobial Resistance Monitoring System (NARMS) (Folster *et al.*, 2009).

*Salmonella* infections caused by imported food and food animals represent an increasingly important cause of human infections in some countries, highlighting the need for better import control and testing. The approach could help limit the number of human infections, but result in considerable challenges for exporting countries and eventually limited the volume of infected food products.

#### *International travel*

In 2004, global travel reached a record of 763 million arrivals, an increase of 11% compared to 2003 ([www.world-tourism.org](http://www.world-tourism.org)). The increase was observed in all regions but was most profound in Asia (28%) and the Middle East (18%). The intensity of international travel is a public health concern since an increasing number of travellers return home with mild or severe infections (Archambault *et al.*, 2006; III; V). In 2008, 3,022 confirmed human salmonellosis cases were reported to Statens Serum Institute. Of these, 706 (23.3%) were confirmed to be travel associated and 95 (13.4%) of these cases were linked to travelling to Thailand (unpublished data). In the same year, 149,570 Danes visited Thailand (<http://www.tourism.go.th/>). The number of infected Danes returning back from Thailand might be underestimated and may have been 10-20 times higher (Wheeler *et al.*, 1999). Taking this underestimation into consideration, 0.6% of the Danes visiting Thailand may have brought back a *Salmonella* infection in 2008 (V). The Swedish database on notifiable communicable diseases identified 24,803 NTS cases associated with

international travel from 1997 to 2003. High risk of disease was seen in travellers returning from Africa, India, and Southeast Asia (Ekdahl *et al.*, 2004).

Three recent studies have linked Danish patients, suffering from either gastrointestinal infections or bacteremia, with travel to Thailand (Archambault *et al.*, 2006; **III**; **V**). One investigation linked six *S. Rissen* isolates recovered from Danish patients with travel to Thailand, where genetically identical *S. Rissen* isolates were found. In addition, several of the isolates shared the same phenotypic and genotypic antimicrobial susceptibility patterns (**III**). Similarly, sporadic cases of travel associated salmonellosis have been frequently described (Collard *et al.*; 2007; Kasper *et al.*; 2009). Recently, an outbreak of travel associated *S. Enteritidis* illness was detected in Finland. Petrov *et al.* linked Finnish outbreak strains with strains from Sunny Beach, Bulgaria where employees at a hotel were also infected. The outbreak likely included tourists from the United Kingdom, Norway, Sweden, and Germany (Petrov *et al.*, 2009).

Multiple studies have revealed that international travel to certain destinations is associated with a relatively high risk of human salmonellosis. However, little has been done to avoid it. The best way to prevent it is ensure an adequate level of food safety globally, and by educating the general population about food borne disease prevention under different circumstances.

#### *International adoptions*

The international adoption of children carrying and transmitting infectious diseases is probably a minor but clearly an overlooked problem. Since 1986, nearly 220.000 children have been adopted by North American families (Miller *et al.*, 2005). In 2007, the most common countries of origin for Danish adoptions were China, Vietnam, South Africa and Ethiopia. For U.S. adoptions, most were from China, Guatemala, Russia and Ethiopia (**IV**). Today, international adoption medicine is a relatively new specialization in paediatrics that has emerged to address the specific health care needs of children and their adoptive families (Miller *et al.*, 2005). Common infectious diseases in adopted children include tuberculosis, hepatitis B and C, HIV, syphilis, parasites and enteric infectious diseases (Miller *et al.*, 2005). In 1997 and 1998, a study determined the prevalence of infectious diseases among 504 adoptees. The data revealed that 90% of the stools were abnormal and that two of the children carried a *Salmonella* species (Saiman *et al.*, 2001).

In France, fourteen *S. Babelsberg* harbouring the *bla*<sub>SHV-12</sub>-like gene and six *S. Enteritidis* isolates were detected in 2002 and 2003 from international adoptees. All of the children were traced back to one orphanage in Mali (Weill *et al.*, 2004).

In 2007, infections caused by *S. Concord* were reported in several countries among children adopted from Ethiopia. A total of 3,419 children were adopted from Ethiopia from 2003 to 2007 and brought to Denmark, England and Wales, Ireland, the Netherlands and the United States.

During this period, the number of children adopted from Ethiopia increased from 221 adoptions in 2003 to 1,385 in 2007. The five countries including Austria reported 78 laboratory confirmed cases of *S. Concord* from 2003 to 2007 (Figure 14) (IV). Simultaneously, another study reported 35 French and 27 Norwegian cases of *S. Concord* of which 28 and 26, respectively, originated in children from Ethiopia (Fabre *et al.*, 2009). In a study by Hendriksen *et al.* where adoption status was known for 44 (79%) of the

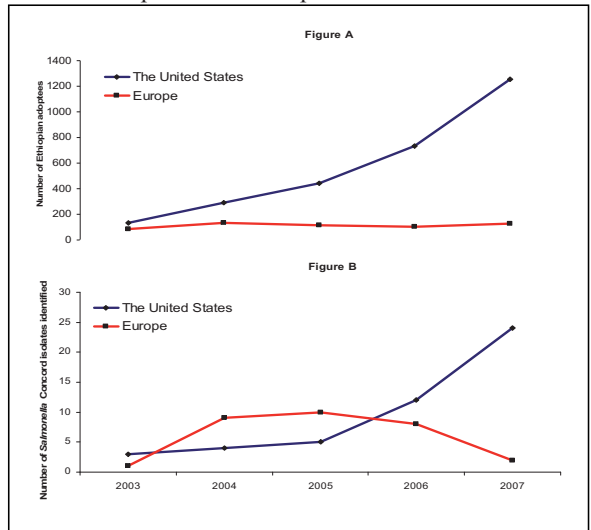


Figure 14. Number of children adopted from Ethiopia (figure A) and number of reported laboratory-confirmed cases of *Salmonella* serotype *Concord* (figure B) per year in Europe and the United States, 2003-2007 (IV).

patients  $\leq 3$  years of age, 43 were adopted from Ethiopia. One patient  $\leq 3$  years of age and two patients  $\geq 18$  years of age were either a sibling or mothers to Ethiopia adoptees (IV). Six adoptees were asymptomatic at the time of adoption. In addition, the data showed that among 35 isolates, all from or associated with a child adopted from Ethiopia, were multi-drug resistant including resistance to third generation cephalosporins. Six (14%) also showed reduced susceptibility to ciprofloxacin. All of the 35 isolates harbored the *bla*<sub>CTX-M-15</sub> gene and 17 of them also the *bla*<sub>SHV-12</sub> gene. Of the six ciprofloxacin resistant isolates three harbored a *qnr* gene (IV). This work indicates the need for mandatory screening of adoptees, not only for foodborne pathogens but also for viruses and parasites, for the benefit of the children and respectively new families. In

addition, the findings should be disseminated to the orphanages along with assistance to resolve the problem of infections.

## SURVEILLANCE

### *Surveillance*

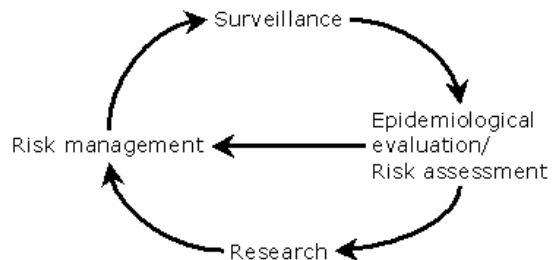
Surveillance is defined by the WHO as a systematic ongoing collection, collation, analysis, and interpretation of data and a timely dissemination of information to those who need to know so that public health actions can be taken (Figure 15).

Surveillance is conducted to facilitate a better control of diseases and lead to

public health actions such as outbreak detection, to measure the magnitude, the burden, and trends of disease, to

improve the knowledge of the disease (causes, sources, reservoirs, risks, morbidity, and mortality), guide programmes to measure the effectiveness of interventions, and to assist policymakers in setting priorities. To date, four generic types of enteric disease surveillance systems exist; no formal (occasional) surveillance, syndromic surveillance, laboratory-based surveillance, and integrated laboratory-based surveillance. The last two types are mostly associated with foodborne pathogens and stand out from the first two types of surveillance systems by being based on laboratory results. Integrated foodborne surveillance refers to concomitant testing of isolates from humans, animals and foods, and may include environmental and animal feed samples.

Surveillance of NTS infections in humans is conducted in a hierarchical structure from local to global. Since 2000, the WHO has volunteered to collect data and facilitate a global surveillance of *Salmonella* by establishing the CDB. Global surveillance is relative important as it render analysis of the major trends worldwide limiting the influence of local outbreaks and other elements which complicate interpretation (II, Galanis *et al.*, 2006). Ongoing overall trend analysis may provide crucial knowledge of emerging serovars on global level which might result



*Figure 15. The cycle of public health protection, illustrating the role of surveillance in supporting risk assessment, risk management and formulating new research efforts. Lo Fo Wong DMA et al., 2004.*

in pandemic proportions and millions of cases or pin out regional endemic serovars. In addition, global surveillance may also identify problems of global importance which may be difficult to detect due to the limitations in national surveillance such as occurrences of important rare serovars in low frequencies present in several countries (IV). Regional or national surveillance are mostly governed by the individual countries and conducted by national or regional public health institutes. Regional or national surveillance allows the scientists to narrow the focus on specific national emerging or rare serovars. The focus offers the opportunity to initiate detailed epidemiological and molecular studies and set priorities for intervention strategies and elucidate associated risk factors (III, V, VI, Bangtrakulnonth *et al.*, 2004, Archambault *et al.*, 2006, Tapalski *et al.*, 2007, Petrov *et al.*, 2009). Local surveillance is often restricted to a confined geographical area such as a large city compared to rural areas. This often results in detection of local minor outbreaks.

#### *Surveillance methods*

In the last decade, our ability to distinguish in a rapid and reliable way between epidemiologically unrelated isolates from the same bacterial species has increased, thereby enhancing our capacity to detect outbreaks, conduct surveillance and understand or elucidate the epidemiology of certain types or clones. Thus, bacterial typing techniques have been developed to measure genetic relatedness among emerging pathogenic strains, clones or clusters of bacteria from a single species.

In the beginning of the bacterial typing era, typing systems were based solely on phenotypic methods such as serotyping (Grimont *et al.*, 2007), phage typing (Smith *et al.*, 1951; Guinee and Scholtens, 1967; Sechter and Gerichter, 1968; Petrow *et al.*, 1974; Rowe *et al.*, 1980; Tyc *et al.*, 1980; Scarlata *et al.*, 1982; Sharma *et al.*, 1984; Sood and Basu, 1984; Ward *et al.*, 1987) and antibiogram typing (Figure 16). These methods are currently in use in many countries as part of national surveillance programmes. The results obtained by these phenotypic tests, particularly serotyping, have been the basis for the elucidation of the epidemiology of NTS for decades. In addition, conventional serotyping and antimicrobial susceptibility testing are excellent surveillance tools and also used as first line methods in outbreak detection caused by NTS. Recently, several DNA fingerprinting and array techniques have been developed serving as an alternative to the conventional serotyping method (McQuiston *et al.*, 2004; Fitzgerald *et al.*,

2007). By merging these two methods one should be able to identify the major serotypes (McQuiston *et al.*, 2004). The method has proved its worth in the characterization of a monophasic variant of *S.*

Goettingen where conventional serotyping was insufficient (Petrov *et al.*, 2009).

Phenotypic methods may not have sufficient discriminatory power in an outbreak situation, and may need to be supplemented with molecular techniques such as PFGE (Raufu *et al.*, 2009; **III**; **IV**; **V**). The number of genotyping methods and their discriminatory power has increased and several methods have been implemented to meet public health needs. The most commonly used genotyping method for surveillance and outbreak detections is PFGE (Tenover *et al.*, 1995; Ribot *et al.*, 2006; van Belkum *et al.*, 2007), however Multi Locus Sequence Typing (MLST) and Multiple Locus Variable Number Tandem Repeat Analysis (MLVA) (Lindstedt *et al.*, 2003; Tapalski *et al.*, 2007; van Belkum *et al.*, 2007; Lindstedt *et al.*, 2008) are also commonly used. In the near future, these methods will likely be superseded by microarray technologies and full genome sequencing (Maslow *et al.*, 1993; Foxman *et al.*, 2005; van Belkum *et al.*, 2007). Since 1984, PFGE has been standardized for *Salmonella* testing, allowing comparison of patterns between laboratories around the world (Ribot *et al.*, 2006). Similarity indexes have partly replaced the criteria by Tenover *et al.* by implementation of the BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium) for interpretation of the PFGE profiles. In the United States, the preferred subtyping method is currently PFGE used by PulseNet

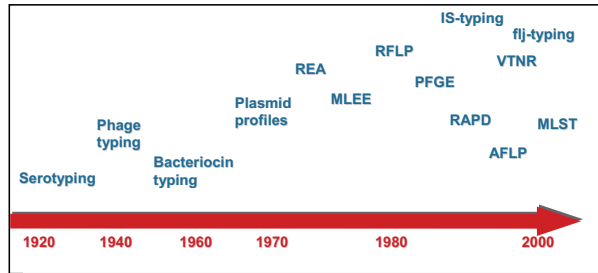


Figure 16. Evolution of typing methods. Courtesy of Branko Kokotovic.

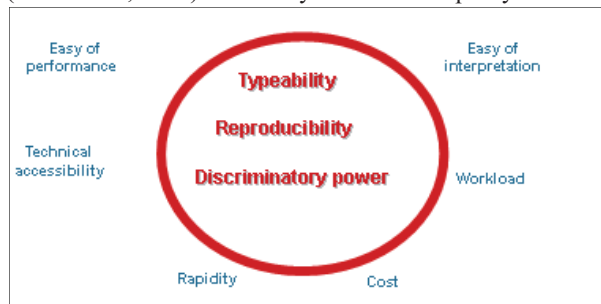


Figure 17. Criteria for evaluation of typing methods. Courtesy of Branko Kokotovic.

USA for outbreak detection (Gerner-Smidt *et al.*, 2006) and it has also shown to be useful in epidemiological studies of NTS worldwide (III; IV; V).

Scientists in PulseNet USA are currently developing a MLVA scheme for both *S. Typhimurium* and *S. Enteritidis*. The strategy is to first integrate these new methods into the PulseNet platform to complement PFGE data (Gerner-Smidt *et al.*, 2006). There is still a need for a common MLVA nomenclature in order to compare results among laboratories. However, a recent publication proposes such a nomenclature that is independent of equipment and primers used (Larsson *et al.*, 2009). Before applying a typing technique, it is important to understand and evaluate the strengths and weaknesses of the methods. Certain criteria are normally used to assess this such as the discriminatory power,

reproducibility, typeability,

and repeatability (Figure 17). A good typing method should

give the same result each time tested in the same laboratory (repeatability) but also when tested in different

laboratories (reproducibility). Typically, sequence-based methods are more repeatable and reproducible than gel based methods (Table 1). At the same time the methods should be able to assign a specific type to all tested isolates (typeability) and furthermore, be able to assign a different type to two epidemiologically unrelated isolates sampled randomly from a population of the same bacterial species (discriminatory power) (Foxman *et al.*, 2005; van Belkum *et al.*, 2007). In addition, several other aspects also affect the choice of typing techniques such as the costs, accessibility, and workload.

### Surveillance programmes

In February 1997, a global survey of the 191 WHO member states was initiated to estimate the number of countries conducting public health surveillance of *Salmonella*. Only 76 (73%) of 104 countries reported conducting *Salmonella* surveillance ranging from all countries in Europe to 40% of the countries in the Western Pacific region. A total of 69 countries among the 76 countries conducting surveillance of *Salmonella* included serotyping (Herikstad *et al.*, 2002).

| Typing technique        | Discriminating power | Repeatability | Reproducibility | Comment                                    |
|-------------------------|----------------------|---------------|-----------------|--|
| Whole genome sequencing | Very High            | High          | Medium / High   |  |
| MLST                    | Medium / High        | High          | Medium / High   | Depends on gene choice                     |
| MLVA                    | High                 | High          | High            |  |
| PFGE                    | Medium / High        | Medium        | Medium          | Depends on type and number of enzyme used. |
| Conventional serotyping | Medium               | Medium        | Medium          |  |

Table 1. Criteria of different typing techniques.

This number is quite low considering that *Salmonella* in many countries is either the first or second most common foodborne bacterial pathogen.

Despite the low number of countries conducting surveillance based on serotyping, some countries have a long tradition for *Salmonella* surveillance or have recently initiated the programmes. One of the countries that stand out as having conducted laboratory-based surveillance for at least 15 years is Thailand. Each year, the National Institute of Health publishes an annual report of laboratory-confirmed *Salmonella* and *Shigella* in Thailand (Bangtrakulnonth *et al.*, 2006). This is one among many reasons why many international *Salmonella* studies have been based on or included Thai data (Bangtrakulnonth *et al.*, 2004; Archambault *et al.*, 2006; Aarestrup *et al.*, 2007; Vindigni *et al.*, 2007; **III**; **V**; **VI**). In addition to the Thai program, several other countries conduct annual *Salmonella* surveillance including New Zealand (Anonymous, 2008a), South Africa (Anonymous, 2008c), the United States (Anonymous, 2008e), Canada (Anonymous, 2008b), Korea, Denmark, Norway, and Sweden.

In the United States, several institutes independently conduct *Salmonella* surveillance in humans, food and animals (Jones *et al.*, 2007). The Centers for Disease Control and Prevention (CDC) collect data on reported laboratory confirmed *Salmonella* cases of human origin to the National *Salmonella* Surveillance System (NSSS) through the Public Health Laboratory Information System (PHLIS). The PHLIS database contains data from all state and territorial public health laboratories (Anonymous, 2008e). The United States Department of Agriculture, Food and Safety Inspection Service (USDA-FSIS) and the United States Food and Drug Administration Center for Veterinary Medicine (FDA-CVM) collect *Salmonella* data from food animals and retail meats, respectively.

Integrated laboratory-based *Salmonella* surveillance systems are established in a small number of countries such as the United Kingdom (Anonymous, 2007f), Australia (OzFoodNet Working Group, 2003), and Denmark (Anonymous, 2009a). In the United States, the surveillance in humans is from all 50 states in contrast to animals, which are from all federally inspected abattoirs (Jones *et al.*, 2007). However, in Canada an integrated surveillance programme for antimicrobial resistance has been established (Anonymous, 2008b) which also includes serovar distribution.

In contrast, only a few developing countries have a *Salmonella* surveillance system implemented and none of these systematically integrates data from humans, food, and animals.



In developing countries, information on foodborne pathogens is limited due to several factors such as patients who do not seek medical care, samples that are not processed and/or reported data to central public health institutes. In Mexico, syndromic surveillance was replaced in 2002 by an integrated laboratory-based surveillance system in four states. The sampling scheme was designed to follow the food chain in a temporal mode. In a test week, food animal intestines were collected on the first sampling day, followed by raw retail meats on the second to the fourth day. From day 7 to 14, fecal samples from asymptomatic children were collected. This surveillance revealed high rates of contaminated meat and ceftriaxone resistant salmonellae. Genotyping data showed 14 PFGE clusters with indistinguishable patterns associated with human, retail meat, and food animals (Zaidi *et al.*, 2008). This approach could be technically feasible for many developing countries for future surveillance efforts. Unfortunately, it is too expensive why the described study is currently not operating.

One could wish that WHO had the power and authority to strengthen through Codex Alimentarius mandatory laboratory-based surveillance of foodborne pathogens in food animals, food and humans in order to gain detailed data for initiating action to prevent human salmonellosis.

#### *A harmonised multi-national integrated laboratory-based surveillance programme*

In 2003, the European Parliament and Council, adopted; Directive 2003/99/EC on the monitoring of zoonoses and zoonotic agents (Anonymous, 2003a; O'Brien *et al.*, 2005) amending Decision 90/424/EEC (Anonymous, 1990) and repealing Council Directive 92/117/EEC (Anonymous, 1992). The purpose of the Directive 2003/99/EC was, among other things, to ensure a proper and harmonised surveillance of zoonoses including *Salmonella*. The Directive was supplemented later the same years with the Regulation 2160/2003 on the control of *Salmonella* and other specified foodborne zoonotic agents, which in principle should cover the whole food chain, from farm to table (breeding flocks of *Gallus gallus*, laying hens, broilers, turkeys, herds of slaughter, pigs, breeding herds of pigs) (Anonymous, 2003b; O'Brien *et al.*, 2005). The sampling should take place over a three year period with the possibility of extension. The surveillance should take place on a harmonised basis by all member states according the detailed rules laid down in the regulation. In addition, all national reference laboratories should participate in proficiency testing arranged by the Community Reference Laboratory (CRL) to insure a high quality of the results

submitted. The data are complied and published annually by the European Food Safety Authority (EFSA) (Anonymous, 2009b) including human data collected by European Centre for Disease Prevention and Control (ECDC) and through the European Surveillance System.

The experience from the European Union has proven how important it is

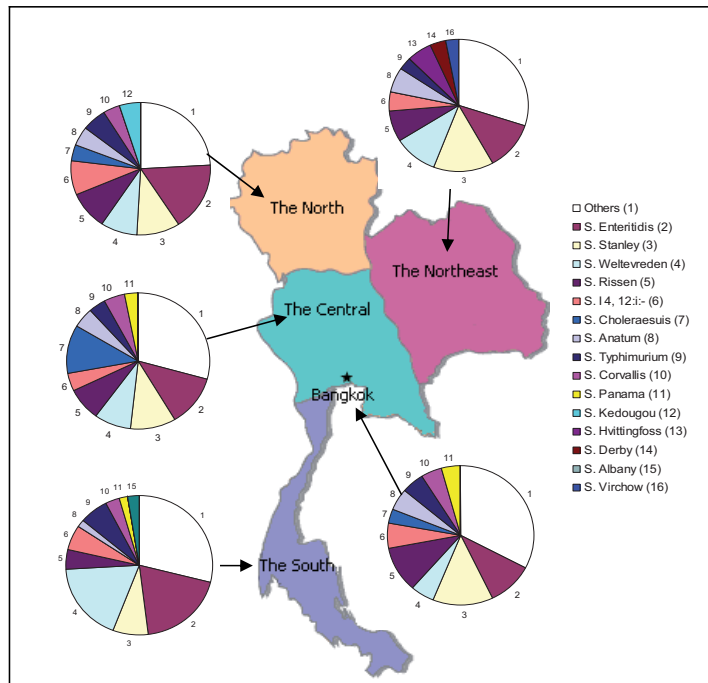


Figure 18. Distribution of the top ten most common serovars in the different regions in Thailand from 2002 -2007. (VI)

to design the surveillance program to include as many of the countries and

the population as possible and to ensure a high quality of the data. Nevertheless, the overall serovar distribution and frequencies in the EFSA report does not necessarily reflect the distribution of serovars in a single country. Several studies have shown large differences in the distribution of NTS between countries but also among regions within a country (V; VI).

Hendriksen *et al.* revealed that the serovar distribution varied considerably among five regions within Thailand as 15 serovars were listed among the top 10 most common serovars in the regions (Figure 18). In Thailand, the distribution of serovars seemed to be culturally linked, and was for instance influenced by differences in pork and poultry consumption between the Buddhist North and the Muslim population in Southern Thailand (VI).

In Thailand, a large amount of data has been generated, but it is not easily accessible.

Nevertheless, harmonizing surveillance programmes will in the future be of high priority in order to have comparable data on occurrence and frequencies between countries and regions, and to

facilitate joint actions directed towards the most important sources of human infections in a region or country.

#### *Outbreak surveillance programmes*

Sporadic cases and outbreaks caused by NTS is often linked to consumption of meat, egg and recently also to contaminated fruits and vegetables (Nygård *et al.*, 2008; Hanning *et al.*, 2009). In the United States, 121 *Salmonella* outbreaks were recorded to the CDC Foodborne Outbreak Reporting System in 2006. The outbreak consisted of more than 3.300 cases and the most common serovars responsible for the outbreaks were *S. Enteritidis* (n=26), *S. Typhimurium* (n=26), *S. Newport* (n=10), and *S. Heidelberg* (n=10) (Anonymous, 2008e). In comparison, there were in 2007 observed six outbreaks caused by *Salmonella* which accounted for 12% of all outbreaks in Denmark. The serovars involved in these outbreaks were *S. Typhimurium* (n=27), *S. Weltevreden* (n=19), *S. Heidelberg* (n=13), *S. Enteritidis* (n=9), and *S. Senftenberg* (n=3) (Anonymous, 2009a). In 2007, a total of 2.201 outbreaks were reported in 22 European countries ranging from five in Ireland and Romania to 843 in Germany. *S. Enteritidis* was by far the most common serovar responsible for the outbreaks (n=355) infecting 5.940 humans. The second most common serovar was *S. Typhimurium* with 63 outbreak (Anonymous, 2009d).

In recognition that infections, including outbreaks, may originate in different countries, the Salm-net project was established in 1993 under the leadership of the Health Protection Agency (HPA). A main goal of Salm-net was to harmonise the *Salmonella* phage typing schemes used in Europe (Fisher *et al.*, 2004; Hald *et al.*, 2004). Participants in the Salm-net project were public health reference laboratories in the European Union, Norway, Switzerland, Australia, Canada, Japan, and South Africa. Salm-gene, another European network established by HPA, began in 2001 to build a database based for *Salmonella* PFGE profiles among European countries. By the end of 2004, the Salm-gene database contained approximately 20.000 profiles of primarily *S. Enteritidis* and *S. Typhimurium* (Swaminathan *et al.*, 2006). In 2004, the Salm-gene network merged with Enter-Net which later became PulseNet Europe in 2004 (O'Brien *et al.*, 2005; Fisher *et al.*, 2005).

In the United States, PulseNet USA was established in 1996 as the national molecular surveillance network for foodborne infections (Gerner-Smidt *et al.*, 2006). The aim of the network was to rapidly detect outbreaks caused by foodborne pathogens by the use of the PFGE.

In 2001, full national participation by all 50 states was achieved. By 2005 the network consisted of 65 participating public health laboratories, four countries, three cities and eight food safety regulatory laboratories. All PFGE profiles are uploaded to the national database where local or multi-state outbreaks are investigated. In 1999, PulseNet USA harmonised protocols with PulseNet Canada forming PulseNet International (Swaminathan *et al.*, 2006). PulseNet USA and PulseNet Canada have investigated numerous multi-national outbreaks, showing the advantage of international collaboration in food safety surveillance. In addition to PulseNet USA and PulseNet Canada, PulseNet networks have been established in the Asia Pacific region, Latin America, Europe, and China. Surveillance by PulseNet Europe ceased in 2007 due to lack of funding, but it is expected that funding from the ECDC will resume in 2010.

## CONTROL, INTERVENTION, AND PREVENTION

### *Analysis of surveillance data*

Surveillance of NTS is not only about collecting data, but also analyzing the data to identify critical points of intervention. Many types of analyses are possible. Case control studies to identify risk factors can be sufficient to focus interventions and prevention measurements. Source attribution modelling can provide detailed information about the nature and magnitude of different reservoirs contributing to infection. Source attribution is partitioning of the human disease burden of one or more foodborne infections to specific sources, guiding authorities to prioritise intervention and control efforts and measuring the impact (Pires *et al.*, 2009). Several factors need to be known to attribute burden to specific sources. A laboratory-based surveillance system must be in place, and the burden of illness determined. In addition, the proportion of foodborne disease due to international travel should be estimated and food items categorised. In 2004, a Danish mathematical model was published for quantifying the number of domestic and sporadic cases caused by different serovars and phage types as a function of the prevalence of the same serovars in each major animal or food sources (Hald *et al.*, 2004). In the following years, the model was enhanced to include information on antimicrobial susceptibility. Using this model, researchers in Denmark were able to quantify the contribution of various animals, foods, and international travel to human infections with resistant NTS (Hald *et al.*, 2007). In the United States, the Foodborne Diseases Active Surveillance Network (FoodNet) adopted the same model to attribute the human *Salmonella* cases to various sources (Gerner-Smidt *et al.*, 2006).

### *Host specific control programmes and interventions*

Denmark has one of the best national programs in the world for controlling and preventing *Salmonella*, and other nations look to Denmark for leadership in this area. In the 1980s, the incidence of salmonellosis in Denmark steadily increased, and was linked to the consumption of broiler chicken and pork. This led to a targeted national control programme (Wegener *et al.*, 2003; Mousing *et al.*, 1997) based on the general prevention strategy known as Hazard Analysis and Critical Control Points (HACCP). HACCP is a systematic preventive approach to food safety that addresses physical, chemical and biological hazards as a means of prevention, rather than finished product inspections. The HACCP strategy is to identify key steps (critical control points) in the chain from farm to fork (Busani *et al.*, 2006) where interventions will have the most impact in reducing or eliminating food safety hazards.

In 1995, the most extensive nation-wide control programme ever attempted was launched in Danish finishing swine herds. All swine herds were tested and categorized based on their *Salmonella* prevalence. Herds were assigned to three categories: herds with low and acceptable prevalence, moderate prevalence, and clearly unsatisfactory prevalence (Wegener *et al.*, 2003). Swine herds belonging to the unsatisfactory category were slaughtered using special hygienic precautions according to the inherent risk. In 2001, the classification scheme was extended to include a fourth category, namely, herds being negative in serological tests (Alban *et al.*, 2002). A similar approach was implemented for poultry. All shell eggs from layer flocks should be free from *S.*

Enteritidis and *S.* Typhimurium, and suspected or confirmed positive eggs should be pasteurized prior to marketing. In addition,

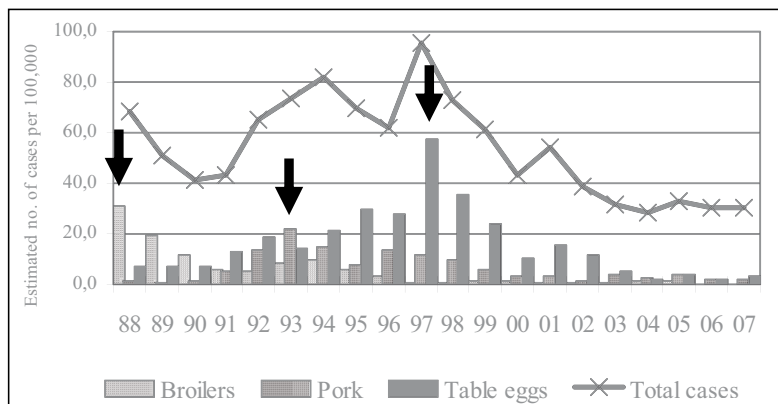


Figure 19. Trends and sources of human salmonellosis in Denmark, 1988 to 2007. Source: Danish Zoonosis Centre, National Food Institute, Technical University of Denmark

infected flocks either were eliminated or slaughtered separately or late in the day to avoid cross-contamination (Wegener *et al.*, 2003; Hald *et al.*, 2005). These control programmes and targeted interventions resulted in a major reduction in the incidence of human salmonellosis in Denmark. The broiler, pork, and egg associated salmonellosis incidences were reduced by 95% (1988 to 2001), 85% (1993 to 2001), and 75% (1997 to 2001), respectively (Figure 19) (Wegener *et al.*, 2003). The benefit of implementing the control programmes by eliminating infected animals and diversifying slaughter were estimated to save the taxpayers in Denmark \$25.5 million annually. In 2003, the European Parliament and Council issued, in combination with the harmonised surveillance system, Regulation 2160 on the control of *Salmonella* and other specified food-borne zoonotic agents. This regulation was intended to control *Salmonella* and other specified foodborne zoonotic agents by reducing transmission from poultry and pigs (Anonymous, 2003b). The regulation enforced strict rules for all Member States to reduce the prevalence of *Salmonella* in primary production. The control programmes target breeding flocks of *Gallus gallus* in 2004, followed by laying hens, broilers, turkeys, slaughter pigs and breeding pigs over the subsequent four years (Anonymous, 2003b).

The increased number of human infections caused by international travel is in many countries a major concern. Currently, there is no vaccine to prevent infections caused by NTS for travellers. Thus, in many countries, public health authorities have initiated campaigns to educate the public on steps to reduce the risk of salmonellosis. Consumers are advised not to eat raw or undercooked eggs, poultry or meat, not to consume raw and unpasteurised dairy products, and to clean vegetables before consumption. The WHO instituted a similar communication programme as part of their global strategy to decrease the burden of foodborne diseases. This led to the WHO report “The Five Keys to Safer Food”, which was published in 2001, and included and associated training materials developed to provide countries with materials that are easy to use, reproduce and adapt to different target audiences ([www.who.int/foodsafety/consumer/5keys/en/](http://www.who.int/foodsafety/consumer/5keys/en/)). Several publications have shown that adopted children carrying *Salmonella* is an overlooked problem (Saiman *et al.*, 2001; Weill *et al.*, 2004; Fabre *et al.*, 2009; **IV**). The American Academy of Paediatrics recommends that a stool specimen be collected from all adopted children entering the United States and cultured for the presence of bacterial pathogens such as *Salmonella* (Hostetter *et al.*, 1991; Nicholson *et al.*, 1992; Stauffer *et al.*, 2002). The utility of this recommendation was highlighted to all countries in a study where family members were infected

with *S. Concord* which was introduced by adopted children (IV). Another approach in preventing travel associated salmonellosis is by publishing data on the burden of salmonellosis, and routes of transmission, in different countries. Thus, urging the country to take action to limit the sources of infections among the general population and travelers by improving the food safety. (Aarestrup *et al.*, 2007; V; VI). However, this might be a challenge due to the limited knowledge of the epidemiology of *Salmonella* in developing countries and the circumstances associated with the production systems, which often are many small operations where animals are reared with minimal oversight such as free range.

#### *Serovar specific control programmes and interventions*

To date, only a few studies describe effective serovars-specific control programmes that do not rely on vaccination. Multidrug resistant *S. Typhimurium* DT104 appeared in the 1980s and became a major cause of salmonellosis around the world. In Denmark, a component to control and prevent *S. Typhimurium* DT104 to spread and cause infections in humans was incorporated into the existing control program for pig herds (Alban *et al.*, 2002). Farmers with *S. Typhimurium* DT104 infected herds had to follow a herd intervention plan which included restrictions on livestock trade and special slurry management. Carcasses of infected pigs had to be heat treated or decontaminated before leaving the abattoir (Nielsen *et al.*, 2001). Likewise, a Danish control programme for *S. Dublin* was launched in 2002 attempting to reduce the increasing number of human infections and the economic losses for the cattle industry, where this serovars was a problem. In contrast to the control programme in pigs, this programme sought to identify cattle herds free of infection by periodic measurement of *S. Dublin* antibody titres in bulk milk and by modelling the spread between herds (Hald *et al.*, 2005; Jordan *et al.*, 2008). The outcome of the modelled control programme showed that restricting the movement of herds between regions was more important than attempting control within herds. However, a combination seems to be more effective but needs to be further explored (Jordan *et al.*, 2008).

#### *Prevention by vaccination*

To date, all attempts to develop a comprehensive vaccine for humans and animals that will cover all important serovars of *Salmonella* have failed. Today, only vaccines for humans against *S. Typhi* exist. One study revealed that a Vi *S. Typhi* vaccine did not protect against *S. Paratyphi* A

or B since these serovars do not express the Vi polysaccharide. However, a Ty21a *S. Typhi* vaccine conferred substantial cross protection against *S. Paratyphi B* but not to *S. Paratyphi A* (Levine *et al.*, 2009). This example highlights the difficulties in developing *Salmonella* vaccines. A major problem is that both serovar-dependent and host-dependent factors, as well as the attributes influencing serovar host specificity, are unknown among the more than 2,500 serovars (Barrow *et al.*, 2007). Some success has been seen with *S. Enteritidis*, which is associated with chickens and is mainly transmitted in eggs. In the United Kingdom, a widespread vaccination program was implemented for egg-laying hens to reduce transmission of *S. Enteritidis*. The programme was successful in achieving a significant decrease of human infections caused solely by *S. Enteritidis* (Gast *et al.*, 2007).

## FUTURE PREDICTIONS AND PERSPECTIVES

Zoonotic infections are responsible for a large and growing proportion of the mortality and morbidity throughout the world. Foodborne zoonoses will likely continue to be important in the future as the global population moves more toward meat products as a source of protein (Merianos *et al.*, 2007; Murphy *et al.*, 2008). In general, the human infections caused by NTS will most likely not significantly decrease in the future unless the global intervention towards eggs succeeds. However, the serovar distribution will probably on a global scale be influenced by increased trade, travel and consumption of exotic food or food produced to low costs (III; IV; V;VI).

Most of the research conducted in the last decades has been focused on *S. Enteritidis* and *S. Typhimurium*, while relatively little is known about the epidemiology of rare serovars with the potential to increase globally.

Recently focus on NTS in the African region revealed that serovars causing gastrointestinal infections also are invasive (Morpeh *et al.*, 2009). We predict to see stronger evidence of NTS causing bacteremia in humans from Africa in time due to the efforts by WHO to implement and enhance *Salmonella* surveillance and burden of illness studies in this area. Another alarming development which lay ahead is the increasing frequency of antimicrobial resistance in NTS. In the last decade, the Western world has tried to minimise the usage of fluoroquinolones and third and fourth generation cephalosporins in both the human and veterinary medicine. These two drug classes are paramount in treating salmonellosis caused by multi-drug resistant strains. Recently,



the WHO has stated that antimicrobial resistance is a global public health concern and has top priority. Evidence shows extensive usage and resistance to fluoroquinolones and third and fourth generation cephalosporins from developing countries, driven in part by low prices and weak or absent use restrictions (Archambault *et al.*, 2006; Aarestrup *et al.*, 2007; Fabre *et al.*, 2009; Lee *et al.*, 2009; II; IV).

In the past two decades, our understanding of *Salmonella* biology and epidemiology has grown tremendously. Advances in technology are moving toward rapid, high-throughput, comprehensive analytical methods. It is highly likely that many, if not all, phenotyping techniques will be supplanted by platforms based on DNA sequence and gene expression tools. To make this transition, it will be necessary to validate the sequenced-based methods to the older phenotypic methods in order to avoid the loss of knowledge and the ability to compare with “historical” data (Hyytiä-Trees *et al.*, 2007). It is expected that within the next decade, microbial genomic sequencing will become inexpensive and routine worldwide. Currently, the technique is faced with a limiting factor of how to assemble, process and handle the large amount of data full genome sequencing will create. Despite of this limitation, software to resolve this problem will most likely be developed in the future making rapid tools for analysis available for extraction of biological and epidemiological data. The data could be applied in multiple ways for typing, genetic comparison or even non-specific vaccines.

Over the past decade, the WHO GFN has promoted capacity building for integrated, laboratory-based surveillance through training courses and activities around the world. The network consists of more than 1200 researchers in more than 700 institutes in 158 member states and 64 training courses have been conducted to date. It is expected that more countries will establish *Salmonella* laboratory-based or integrated laboratory-based surveillance. One challenge is to ensure international harmonization of surveillance systems so that data can be directly compared. Despite of the good intention for conducting surveillance in a global context the benefit for developing countries might be controversial. *Salmonella* infections in developing countries are frequently endemic resulting in a high incidence of symptomatic infections primarily in children causing immunity. Food consumption is often based on locally produced food and with the acquired immunity it is likely to result in much fewer outbreaks compared to e.g. the United States. It will require huge investments in sanitation infrastructure as a whole to decrease the

burden of *Salmonella* in these countries in contrast to a recall of a specific food product in a developed country (Zaidi *et al.*, 2009).

The recent development of source attribution models and the increased number of developed countries conducting integrated laboratory-based surveillance will most certainly results in more countries attributing human infections to various sources. An increased number of countries performing source attribution will probably enable epidemiologists to target control and prevention programmes to the principal reservoirs and serovars responsible for the majority of salmonellosis cases in a global context to avoid a new pandemic. In addition, applied research linking across the human, livestock and food products is needed to increase preparedness planning and the development of evidence-based approaches to zoonotic disease prevention and control (Merianos *et al.*, 2007). Therefore, long term planning that takes into consideration the unique nature of zoonosis is needed.

## CONCLUSIONS AND RECOMMENDATIONS

The studies included in this thesis have confirmed the importance of international travel and consumption of imported food for NTS infections in humans. Globally, the serovars *S. Enteritidis* and *S. Typhimurium* seems to decrease in relative importance whereas a large diversity of other non-specific serovars are increasing; indicating that serovar specific interventions for *S. Enteritidis* and *S. Typhimurium* are ineffective against other NTS. Reliable serotyping data revealed large differences among commonly isolated serovars between continents and less difference between countries within the same continent. Global surveillance identified common problems in several countries, which then was elucidated by detailed epidemiological and molecular studies. Country specific studies have also revealed differences between regions within a country associated with specific risk factors. The thesis illustrates the value of global harmonised surveillance in detecting global trends and identifying local and global problems which then were elucidated. The thesis also illustrates the great value of combining epidemiology and molecular microbiology. There is still a huge lack of knowledge regarding the global epidemiology of *Salmonella*. There is a global need for implementing timely systematic integrated laboratory-based surveillance for *Salmonella* in combination with extensive collection of epidemiological data to target prevention and intervention strategies to diminish the worldwide burden of human salmonellosis.

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## Article I

**WHO Global Salm-Surv External Quality Assurance System (EQAS) for serotyping of  
*Salmonella* isolates, 2000 - 2007.**

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## WHO Global Salm-Surv External Quality Assurance System for Serotyping of *Salmonella* Isolates from 2000 to 2007<sup>▽</sup>

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An international external quality assurance system (EQAS) for the serotyping of *Salmonella* species was initiated in 2000 by WHO Global Salm-Surv to enhance the capacity of national reference laboratories to obtain reliable data for surveillance purposes worldwide. Seven EQAS iterations were conducted between 2000 and 2007. In each iteration, participating laboratories submitted serotyping results for eight *Salmonella* isolates. A total of 249 laboratories in 96 countries participated in at least one EQAS iteration. A total of 756 reports were received from the participating laboratories during the seven EQAS iterations. Cumulatively, 76% of participating laboratories submitted data for all eight strains, and 82% of strains were correctly serotyped. In each iteration, 84% to 96% of the laboratories correctly serotyped the *Salmonella enterica* serovar Enteritidis isolate that was included as an internal quality control strain. Regional differences in performance were observed, with laboratories in Central Asia and the Middle East performing less well overall than those in other regions. Errors that resulted in incorrect serovar identification were typically caused by difficulties in the detection of the phase two flagellar antigen or in differentiation within antigen complexes; some of these errors are likely related to the quality of the antisera available. The results from the WHO Global Salm-Surv EQAS, the largest of its kind in the world, show that most laboratories worldwide are capable of correctly serotyping *Salmonella* species. However, this study also indicates a continuing need for improvement. Future training efforts should be aimed at enhancing the ability to detect the phase two flagellar antigen and at disseminating information on where to purchase high-quality antisera.

*Salmonella* species are among the most important food-borne pathogens, leading to millions of cases of diarrheal illness and thousands of hospitalizations and deaths worldwide each year (3, 7).

More than 2,500 serovars of *Salmonella enterica* have been identified; most human infections are caused by a limited number of serovars. In many developed countries, *Salmonella enterica* serovars Typhimurium and Enteritidis are the most common causes of human salmonellosis (5, 6, 8).

In other regions, however, other serovars have been reported to be more prevalent (1, 3, 5). Changes in the prevalences of specific serovars can result from the movements of people, animals, and food. Correct serotyping is essential for discerning such changes and therefore is essential for efficient outbreak detection and response resulting from laboratory-based surveillance.

In January 2000, the World Health Organization (WHO) launched WHO Global Salm-Surv, a global effort to enhance the laboratory-based surveillance of *Salmonella* infections and

other food-borne diseases and to promote prevention and control activities. Enhancing worldwide serotyping of *Salmonella* species is a key objective of WHO Global Salm-Surv and is facilitated by bench training at international training courses.

To ascertain the performance of participating laboratories and thereby promote enhanced laboratory-based surveillance, an external quality assurance system (EQAS) was established as a part of the WHO Global Salm-Surv program in 2000. Since then, the WHO Global Salm-Surv EQAS has grown to be the largest of its kind worldwide (4, 9). Among other activities, the EQAS conducts an assessment of the capacities of laboratories to correctly serotype *Salmonella* species by shipping eight blinded *Salmonella* isolates for serotyping. Iterations of the EQAS are organized yearly by Denmark's National Food Institute (DTU Food) in collaboration with WHO, the U.S. Centers for Disease Control and Prevention (CDC), and France's Institut Pasteur. The WHO Global Salm-Surv EQAS is a self-evaluating system: after submitting their results to the EQAS Web-based reporting system via a secured individual log-in pass code, participants receive a report that itemizes errors relative to the expected results. The report is intended to be used by the participants for evaluating the accuracy of current techniques and the quality of antisera. The goal is to have all laboratories perform serotyping of *Salmonella* with a

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maximum of one error out of eight isolates. Here we report the results of the first seven iterations of the WHO Global Salm-Surv EQAS *Salmonella* serotyping procedures, conducted from 2000 to 2007. In 2005, no iteration was conducted, due to an internal assessment of the system.

## MATERIALS AND METHODS

Participation in the WHO Global Salm-Surv EQAS is open to all laboratories free of charge. An invitation to participate in each EQAS iteration was announced through Electronic Discussion Group messages, which are received by WHO Global Salm-Surv members. All members receive these messages by e-mail or by fax. Previous messages are archived on the WHO Global Salm-Surv website ([http://www.who.int/salmsurv/activities/bulletin\\_board/en/](http://www.who.int/salmsurv/activities/bulletin_board/en/)). In 2007, WHO Global Salm-Surv membership included >1,000 microbiologists and epidemiologists from 152 countries representing >140 national reference laboratories (animal, food, or public health). Scientists who attended WHO Global Salm-Surv international training courses were automatically registered as WHO Global Salm-Surv members. Through 2008, 56 training courses were held at 17 training sites worldwide. The training curricula provided in these courses typically include both classroom and laboratory instructional modules on the identification and serotyping of *Salmonella* species.

Eight *Salmonella* strains, based on the global prevalence of the serotypes, were selected for each EQAS iteration (3). Strains were obtained from the isolate collection of DTU Food. The same strain of *Salmonella* serovar Enteritidis was included as an internal quality control in 2000, 2001, 2004, 2006, and 2007. For the 2002 iteration, an alternate but phenotypically identical strain of *Salmonella* serovar Enteritidis was chosen. All other strains were included only once in the EQAS iterations. Some serovars (e.g., *Salmonella* serovar Typhimurium) were utilized in multiple iterations; however, these strains had different antimicrobial resistance profiles (Table 1).

All *Salmonella* isolates included in the EQAS were serotyped at DTU Food, the CDC, and Institut Pasteur; the serotypes obtained served as the reference standard. O (somatic) and H (flagellar) antigens were characterized by agglutination with hyperimmune sera, and serotypes were assigned according to the Kauffmann-White scheme (11).

Testing instructions and a "participating laboratory record sheet" (PLRS) were copied to a compact disc, enclosed with the *Salmonella* agar stab cultures in double-pack containers (class UN 6.2), and sent to the participating laboratories according to the International Air Transport Association regulations as "Biological Substance Category B," classified as UN 3373. Prior to shipping, each participating laboratory was notified of the shipping arrangements that had been made for the parcels and was given the airway bill number to enable it to track the package and pick it up from the airport. Import permits were necessary for shipping the parcels to several countries.

WHO Global Salm-Surv EQAS participation was free of charge, but each participating laboratory was expected to cover the expenses associated with its testing of the strains in its facility. Participating laboratories were provided with instructions for the initial subculture of the *Salmonella* strains. Participating laboratories serotyped the isolates using protocols routinely utilized in their institutions; therefore, instructions for serotyping the isolates were not provided. Laboratories were required to submit results by uploading the PLRS onto the WHO Global Salm-Surv website or by submitting the completed PLRS by fax to DTU Food.

After submitting results, each participating laboratory received an individual report. Laboratories that submitted results via the website received an instant report via the secure website, and laboratories that sent the results by fax or e-mail received the report using media. The individual reports included all errors and suggestions on how to either solve or investigate the problem. Errors are defined as results that are different from the expected serotypes. Errors were reported as incorrect results only, and no attempt was made to quantify their severity. Fisher exact tests were performed to assess the significance of the observed changes in correct serotyping results and reported errors. Laboratory participation over the years was assessed by logistic regression, and a *P* value of <0.05 was regarded as significant for all statistical tests. SAS Enterprise Guide software (version 3.0; SAS Institute, Cary, NC) was used for the statistical analyses.

## RESULTS

A total of 249 laboratories in 97 countries participated in at least one of the seven iterations of EQAS from 2000 to 2007; 44 laboratories in 35 countries participated in 2000, 96 laboratories in 55 countries in 2001, 99 laboratories in 61 countries in 2002, 127 laboratories in 72 countries in 2003, 127 laboratories in 71 countries in 2004, 130 laboratories in 66 countries in 2006, and 140 laboratories in 68 countries in 2007. The average number of participating laboratories per EQAS iteration between 2000 and 2007 was 102. One hundred twenty-five laboratories participated in three or more iterations, and 92 laboratories participated in four or more iterations. The participating laboratories included national reference laboratories; veterinary, food, and regional public health laboratories; and clinical laboratories. One or more institutions from the countries listed in Table 2 participated in at least one of the EQAS iterations.

The percentage of participating laboratories that performed serotyping on all eight strains during the seven iterations ranged from 54% to 92%, with an average of 76% (Table 3). The percentage of participating laboratories that correctly serotyped the *Salmonella* serovar Enteritidis isolate that was included in six of the seven iterations increased over the years, although the increase was not statistically significant (*P* = 0.37), from 92% (2000) to 96% (2007).

The percentage of correct serotyping results oscillated in the initial years, decreasing from 76% in 2000 to 72% in 2001 and rising to 91% in 2002. In the 2003 cycle, the percentage of correct serotyping results was 80%, and since then it has increased annually, reaching 88% in 2007. Overall, logistic regression indicates a significant increase in the percentage of correct serotyping results over the years (*P* < 0.01), and the average percentage of correctly serotyped isolates across all 7 years was 82% (Table 3).

The goal of the EQAS program is for all participating laboratories to perform *Salmonella* serotyping with a maximum of one error. A total of 756 PLRSs were received during the seven EQAS iterations. The percentage of laboratories reaching the threshold of reporting one or zero errors increased significantly (*P* = 0.04), from 48% in 2000 to 68% in 2007 (data not shown). In addition, the 2007 iteration was the first in which every participating laboratory correctly identified at least one strain.

A wide range of incorrect results was observed across the seven-cycle study period. The rate of errors ranged from 3.6% (2007) to 41.0% (2006). The rate of errors also differed widely between the different isolates within a single year. For example, in 2001, rates of incorrect results for a single isolate ranged from 9.6% for the *Salmonella* serovar Typhimurium isolate to 38.0% for a *Salmonella* serovar Kottbus isolate.

A *Salmonella enterica* subsp. *enterica* serovar 4,5,12:i:– isolate included in the 2006 iteration accounted for the greatest number of incorrect results (Table 1). A total of 38 laboratories (31.1%) incorrectly serotyped this isolate as *Salmonella* serovar Typhimurium. *Salmonella enterica* subsp. *enterica* serovar 4,5,12:i:– is a common monophasic variant in both Europe and North America. Characterization of other *Salmonella enterica* subsp. *enterica* serovar 4,5,12:i:– strains suggests that these strains are most likely variants of *Salmonella* serovar

TABLE 1. List of *Salmonella* serotypes and errors (EQAS, 2000 to 2007)

| Yr   | WHO no. | Correct serotype               | Correct formula             | No. of labs serotyping | % Errors | Deviating serotype (formula) (no. of labs) <sup>a</sup>   |
|------|---------|--------------------------------|-----------------------------|------------------------|----------|---|
| 2007 | 7.1     | Concord                        | 6,7:l,v:1,2                 | 125                    | 19.2     | Mkamba (I 6,7:l,v:1,6) (5), Potsdam (I 6,7,14:l,v:e,n,z <sub>15</sub> ) (4), Colorado (I 6,7:l,w:1,5), Bonn (I 6,7:l,v:e,n,x), Gabon (I 6,7:l,w:1,2), Kortrijk (I 6,7:l,v:1,7), Langeveld (I 6,7:l,w:e,n,z <sub>15</sub> ), Nessziona (I 6,7:l,z <sub>15</sub> :1,5), Ohio (I 6,7,14:b:l,w), Oakland (I 6,7:z:1,6,[7]), Panama (I 1,9,12:l,v:1,5), Richmond (I 6,7:y:1,2), Strathcona (I 6,7:l,z <sub>13</sub> , z <sub>28</sub> :1,7), Thompson (I 6,7,14:k:1,5), Virchow (I 6,7,14:r:1,2), Wil (I 6,7:d:1,z <sub>13</sub> , z <sub>28</sub> ), <i>Salmonella</i> spp.   |
| 2007 | 7.2     | Enteritidis                    | 9,12:g,m:–                  | 140                    | 3.6      | Blegdam (I 9,12:g,m,q:–), Dublin (I 1,9,12:[Vi]:g,p:–), Potsdam (I 6,7,14:l,v:e,n,z <sub>15</sub> ), Rissen (I 6,7,14:f,g:–), Warragul (I 1,6,14,[25]:g,m:–)  |
| 2007 | 7.3     | Livingstone                    | 6,7:d:l,w                   | 128                    | 10.9     | Kambole (I 6,7,d:l:[2],7) (3), Gabon (I 6,7:l,w:1,2) (2), Paratyphi C (I 6,7,[Vi]:c:1,5) (2), Gombe (I 6,7,14:d:e,n,z <sub>15</sub> ), Herston (I 6,8:d:e,n,z <sub>15</sub> ), Isangi (I 6,7,14:d:1,5), Kisii (I 6,7:d:1,2), Nieverkirk (I 6,7,14:d,z <sub>6</sub> ), Ohio (I 6,7,14:b:l,w), Typhi (I 9,12,[Vi]:d:–)  |
| 2007 | 7.4     | Montevideo                     | 6,7:g,m,s:–                 | 131                    | 6.9      | <i>Salmonella enterica</i> subsp. II (3), Chincol (I 6,8:g,m,[s]:[e,n,x]), Eboko (I 6,8:b:1,7), Menston (I 6,7,g,s:[t]:[i],6]), Othmarschen (I 6,7,14:g,m,[t]:–), Rissen (I 6,7,14:f,g:–)   |
| 2007 | 7.5     | Mbandaka var. 14+              | 6,7,14:z10:e,n,z15          | 131                    | 14.5     | Braenderup (I 6,7,14:e,h:e,n,z <sub>15</sub> ) (6), Djugu (I 6,7:z <sub>10</sub> :e,n,x) (2), Aequatoria (I 6,7:z <sub>4</sub> ,z <sub>23</sub> :e,n,z <sub>15</sub> ), Denver (I 6,7:a:e,n,z <sub>15</sub> ), Georgia (I 6,7:b:e,n,z <sub>15</sub> ), Glostrup (I 6,8:z <sub>10</sub> :e,n,z <sub>15</sub> ), Gombe (I 6,7,14:d:e,n,z <sub>15</sub> ), Kaduna (I 6,7,14:c:e,n,z <sub>15</sub> ), Kastrup (I 6,7:e,n,z <sub>15</sub> :1,6), Larose (I 6,7,g,z <sub>51</sub> :e,n,z <sub>15</sub> ), Lockleaze (I 6,7,14:b:e,n,x), Montevideo (I 6,7,14:g,m,[p]:s:[1,2,7]), Papuana (I 6,7:r:e,n,z <sub>15</sub> ) |
| 2007 | 7.6     | Elisabethville                 | 3,10:r:1,7                  | 130                    | 10.0     | Welltevreden (I 3,10,[15]:r,z <sub>6</sub> ) (5), Simi (I 3,10:r:e,n,z <sub>15</sub> ) (2), <i>Salmonella</i> spp. (2), Give (I 3,10,[15], [15,34]:[d],l,v:1,7), Montevideo (I 6,7,14:g,m,[p]:s:[1,2,7]), Seegefeld (I 3,10:r,i:1,2), Westhampton (I 3,10,[15], [15,34]:g,s,t:–)  |
| 2007 | 7.7     | Poona                          | 13,22:z:1,6                 | 121                    | 14.0     | Bristol (I 13,22:z:1,7) (2), Farmsen (I 13,23:z:1,6) (2), <i>Salmonella</i> spp. (2), Borbeck (I 13,22:l,v:1,6), Derby (I 1,4,[5],12:f,g:[1,2]), Durban (I 9,12:a:e,n,z <sub>15</sub> ), Gabon (I 6,7:l,w:1,2), Kuru (I 6,8:z:l,w), Manhattan (I 6,8:d:1,5), Marburg (I 13,23:k:–), Montevideo (I 6,7,14:g,m,[p]:s:[1,2,7]), Nyanza (I 11:z <sub>4</sub> ,z <sub>26</sub> ), Saugus (I 40:b:1,7), <i>Salmonella</i> subsp. II   |
| 2007 | 7.8     | Isangi                         | 6,7:d:1,5                   | 136                    | 13.2     | Kisii (I 6,7:d:1,2) (4), Kambole (I 6,7,d:l:[2],7) (2), Livingstone (I 6,7,14:d:l,w) (2), Paratyphi C (I 6,7,[Vi]:c:1,5) (2), Wil (I 6,7:d:l,z <sub>13</sub> ,z <sub>28</sub> ) (2), Choleraesuis (I 6,7:c:1,5), Herston (I 6,8:d:e,n,z <sub>15</sub> ), Manhattan (I 6,8:d:1,5), Nieverkirk (I 6,7,14:d,z <sub>6</sub> ), Poitiers (I 6,7:z:1,5), <i>Salmonella</i> spp.   |
| 2006 | 6.1     | <i>Salmonella</i> I 4,5,12:i:– | 1,4,12:i:–                  | 122                    | 41.0     | Typhimurium (I 1,4,[5],12:i:1,2) (38), Farsta (I 4,5:i:e,n,x) (3), Gloucester (I 1,4,12,27:i:l,w) (2), Agama (I 4,12:i:1,6), Lagos (I 1,4,[5],12:i:1,5), Mathura (I 9,46:i:e,n,z <sub>15</sub> ), Paratyphi B (I 1,4,[5],12:b:1,2), Tsevie (I 4,12:i:e,n,z <sub>15</sub> ), Tumodi (I 1,4,12,27:[z <sub>15</sub> ,z <sub>28</sub> :1,5]), <i>Salmonella</i> subsp. V  |
| 2006 | 6.2     | Saintpaul                      | 1,4,12:e,h:1,2              | 119                    | 11.8     | Sandiego (I 1,4,[5],12:e,h:e,n,z <sub>15</sub> ) (6), Chester (I 1,4,[5],12:e,h:e,n,x) (2), Reading (I 1,4,[5],12:e,h:1,5), Bardo (I 8,e,h:1,2), Typhimurium (I 1,4,[5],12:i:1,2), Paratyphi B (I 1,4,[5],12:b:1,2), Chartres (I 1,4,12:e,h:l,w), I 4,12:–:– <sup>b</sup>   |
| 2006 | 6.3     | Virchow                        | 6,7:r:1,2                   | 121                    | 9.9      | Nigeria (I 6,7:r:1,6) (4), I 6,7:r:– (3), Bsilla (I 6,8:r:1,2), Infantis (I 6,7,14:r:1,5), Lomita (I 6,7:e,h:1,5), Papuana (I 6,7:r:e,n,z <sub>15</sub> ), Ngili (I 6,7:z <sub>10</sub> :1,7)   |
| 2006 | 6.4     | Rissen                         | 6,7:f,g:–                   | 118                    | 11.0     | Montevideo (I 6,7,14:g,m,[p]:s:[1,2,7]) (5), Eingedi (I 6,7:f,g:t:1,2,7) (2), Othmarschen (I 6,7,14:g,m,[t]:–) (2), Blegdam (I 9,12:g,m,q:–), Derby (I 1,4,[5],12:f,g:[1,2]), Oranienburg (I 6,7,14:m,t:[z <sub>57</sub> ]), Sandow (I 6,8:f,g:e,n,z <sub>15</sub> )  |
| 2006 | 6.5     | Reading                        | 4,5,12:e,h:1,5              | 121                    | 16.5     | Saintpaul (I 1,4,[5],12:e,h:1,2) (6), Sandiego (I 1,4,[5],12:e,h:e,n,z <sub>15</sub> ) (3), Chester (I 1,4,[5],12:e,h:e,n,x) (2), Bradford (I 4,12,27:r:1,5), Derby (I 1,4,[5],12:f,g:[1,2]), Enteritidis (I 1,9,12:[f]g,m,[p]:[1,7]), Eppendorf (I 1,4,12,27:d:1,5), Hato (I 1,4,[5],12:g,m,s:–), Mono (I 4,12:l,w:1,5), Paratyphi B (I 1,4,[5],12:b:1,2), Typhimurium (I 1,4,[5],12:i:1,2), I 4,5,12:–:– <sup>b</sup>   |
| 2006 | 6.6     | Enteritidis                    | 9,12:g,m:–                  | 124                    | 6.5      | Gallinarum (I 1,9,12:–:–) (2), Berta (I 1,9,12:[f]g,[t]:–), Blegdam (I 9,12:g,m,q:–), Bourmout (I 9,12:e,h:1,2), London (I 3,10,[15]:l,v:1,6), Montevideo (I 6,7,14:g,m,[p]:s:[1,2,7]), Typhi (I 9,12,[Vi]:d:–)   |
| 2006 | 6.7     | London                         | 3,10:l,v:1,6                | 111                    | 9.9      | Amherstiana (I 8:l,v:1,6) (2), Give (I 3,10,[15][15,34]:[d],l,v:1,7) (2), Birmingham (I 3,10,d:l,w), Clackamas (I 4,12:l,v:1,6), Nchanga (I 3,10,[15]:l,v:1,2), Ruzizi (I 3,10:l,v:e,n,z <sub>15</sub> ), Sinstorf (I 3,10:l,v:1,5), Stockholm (I 3,10,[15]:y,z <sub>6</sub> ), Suberu (I 3,10:g,m:–)   |
| 2006 | 6.8     | Give                           | 3,10:l,v:1,7                | 114                    | 9.6      | London (I 3,10,[15]:l,v:1,6) (4), Nchanga (I 3,10,[15]:l,v:1,2) (2), Amsterdam (I 3,10,[15][15,34]:g,m,s:–), Bredeney (I 1,4,12,27:l,v:1,7), Kortrijk (I 6,7:l,v:1,7), Mokol (I 3,10:y:1,7), Stormont (I 3,10:d:1,2)  |
| 2004 | 5.1     | Give                           | 3,10:l,v:1,7                | 111                    | 26.1     | London (I 3,10,[15]:l,v:1,6) (9), Parkroyal (I 1,3,19:l,v:1,7) (3), Meleagridis (I 3,10,[15][15,34]:e,h:l,w) (3), Joal (I 3,10,l,z <sub>28</sub> :1,7) (2), Nchanga (I 3,10,[15]:l,v:1,2) (2), Anatum (I 3,10,[15][15,34]:e,h:1,6), Assinie (I 3,10,l,w,z <sub>6</sub> ), Cannonhill (I 3,10,[15]:19:y:e,n,x), Elisabethville (I 3,10:r:1,7), Litchfield (I 6,8:l,v:1,2), Newbrunswick <sup>b</sup> Nyborg (I 3,10,[15]:e,h:1,7), Ruzizi (I 3,10:l,v:e,n,z <sub>15</sub> ), Sinchew (I 3,10:l,v:z <sub>35</sub> ), Sinstorf (I 3,10:l,v:1,5)  |
| 2004 | 5.2     | Braenderup var. 14+            | 6,7,14:eh:enz <sub>15</sub> | 110                    | 6.8      | Larochelle (I 6,7:e,h:1,2) (2), Norwich (I 6,7:e,h:1,6) (2), Larose (I 6,7,g,z <sub>51</sub> :e,n,z <sub>15</sub> ), Lomita (I 6,7:e,h:1,5), Sanjuan (I 6,7:a:1,5)  |

Continued on following page



TABLE 1—Continued

| Yr   | WHO no. | Correct serotype        | Correct formula                         | No. of labs serotyping | % Errors | Deviating serotype (formula) (no. of labs) <sup>a</sup>   |
|------|---------|-------------------------|---|------------------------|----------|---|
| 2004 | 5.3     | Corvallis               | 8,20:z <sub>4</sub> z <sub>23</sub> :-  | 88                     | 20.5     | Chailey (I 6,8;z <sub>4</sub> z <sub>23</sub> [e,n,z <sub>15</sub> ]) (5), Dabou (I 8,20;z <sub>4</sub> z <sub>23</sub> :l,w) (4), Albany (I 8,20;z <sub>4</sub> z <sub>23</sub> :-) (3), Ackwepe (I 9,46:l,w:-), Breda (I 6,8;z <sub>4</sub> z <sub>23</sub> :e,n,x), Hindmarsh (I 8,20:r:1,5), Kallo (I 6,8:k:1,2), Kentucky (I 8,20:iz <sub>6</sub> ), Noya (I 8:r:1,7)  |
| 2004 | 5.4     | Heidelberg              | 4,12:r:1,2                              | 116                    | 16.3     | Magumeri (I 1,6,14,25:e,h:1,6) (2), Remo (I 1,4,12,27:r:1,7) (2), Typhimurium (I 1,4,[5],12:i:1,2) (2), Winneba (I 4,12:r:1,6) (2), Africana (I 4,12:r:1,d,w), Agona (I 1,4,[5],12:f,g,s:[1,2]), Fayed (I 6,8:l,w:1,2), Hidalgo (I 6,8:r:[i]e,n,z <sub>15</sub> ), Kalamu (I 1,5,[5],12:z <sub>4</sub> z <sub>23</sub> :[1,5]), Kiel (I 1,2,12:g,p:-), Ljubljana (I 4,12,27:k:e,n,x), Paratyphi B (I 1,4,[5],12:b:1,2), Saintpaul (I 1,4,[5],12:e,h:1,2), Sandiego (I 4,[5],12:e,h,e,n,z <sub>15</sub> ), Schwarzengrund (I 1,4,12,27:d:1,7)  |
| 2004 | 5.5     | Chester                 | 4,5,12:eh:enx                           | 110                    | 40.0     | Sandiego (I 4,[5],12:e,h,e,n,z <sub>15</sub> ) (26), Saintpaul (I 1,4,[5],12:e,h:1,2) (5), Abortusequi (I 4,12:-:e,n,x) (3), Agona (I 1,4,[5],12:f,g,s:[1,2]), Goldcoast (I 6,8:r:l,w), Haifa (I 1,4,[5],12:z <sub>10</sub> -1,2), Kaapstad (I 4,12:e,h:1,7), Magumeri (I 1,6,14,25:e,h:1,6), Paratyphi B (I 1,4,[5],12:b:1,2), Sarajane (I 1,4,[5],12,27:d:e,n,x) Texas (I 4,[5],12:k:e,n,z <sub>15</sub> )  |
| 2004 | 5.6     | Corvallis               | 8,20:z <sub>4</sub> z <sub>23</sub> :-  | 87                     | 23.0     | Albany (I 8,20;z <sub>4</sub> z <sub>23</sub> :-) (5), Dabou (I 8,20;z <sub>4</sub> z <sub>23</sub> :l,w) (4), Hailey (I 6,8;z <sub>4</sub> z <sub>23</sub> [e,n,z <sub>15</sub> ]) (3), Bellevue (I 8;z <sub>4</sub> z <sub>23</sub> :1,7) (2), Rechovot (I 8,20:e,h:z <sub>6</sub> ) (2), Newport (I 6,8,20:e,h:1,2[z <sub>6</sub> ]) (2), Bardo (I 8:e,h:1,2), Bovismorbificans (I 6,8,20:r:[i]:1,5)   |
| 2004 | 5.7     | Mbandaka                | 6,7,:z <sub>10</sub> :enz <sub>15</sub> | 104                    | 20.2     | Djugu (I 6,7;z <sub>10</sub> :e,n,x) (7), Menden (I 6,7;z <sub>10</sub> :1,2) (4), Redba (I 6,7;z <sub>10</sub> :z <sub>6</sub> ) (2), Gabon (I 6,7:l,w:1,2), Infantis (I 6,7,14:r:1,5), Lindenburg (I 6,8:i:1,2), Lockleaze (I 6,7,14:b:e,n,x), Namibia (I 6,7:e:e,n,x), Omuna (I 6,7,z <sub>10</sub> :z <sub>35</sub> ), Paratyphi C (I 6,7,[Vi]:c:1,5), Thompson (I 6,7,14:k:1,5)  |
| 2004 | 5.8     | Enteritidis             | 9,12:gm:-                               | 120                    | 5.8      | Blegdam (I 9,12,g,m,q:-) (3), Berta (I 1,9,12:[f]g,[i]:-), Gallinarum (I 1,9,12:-,-), Goverdhan (I 9,12:k:1,6), Typhi (I 9,12:[Vi]:d:-)   |
| 2003 | 4.1     | Montevideo              | 6,7,g,m,s:-                             | 119                    | 10.9     | <i>Salmonella</i> subsp. II (4), Menston (I 6,7,g,s:[i]:1,6) (3), Oakland (I 6,7:z:1,6,[7]), Oranienburg (I 6,7,14:m,t:[z <sub>6</sub> ]) (2), Riggil (I 6,7,g,[i]:-), Rissen (I 6,7,14:f,g:-), Sanjuan (I 6,7:a:1,5), Schwarzengrund (I 1,4,12,27:d:1,7)   |
| 2003 | 4.2     | Schwarzengrund          | 4,12:d:1,7                              | 119                    | 15.1     | Stanley (I 1,4,[5],12,27:d:1,2) (5), Ayinde (I 1,4,12,27:d:z <sub>6</sub> ) (2), Ahmadi (I 1,3,19:d:1,5), Brezany (I 1,4,12,27:d:1,6), Duisburg (I 1,4,12,27:d:e,n,z <sub>15</sub> ), Eppendorf (I 1,4,12,27:d:1,5), Kambala (I 1,4,2:c:z <sub>6</sub> ), Magumeri (I 1,6,14,25:e,h:1,6), Mons (I 1,4,12,27:d:l,w), Montevideo (I 6,7,14:g,m,[p]s:[1,2,7]), Sarajane (I 1,4,[5],12,27:d:e,n,x), Southampton (I 4,12,27:r:z <sub>6</sub> ), Typhimurium (I 1,4,[5],12:i:1,2)   |
| 2003 | 4.3     | Paratyphi B (var. Java) | 1,4,5,12:b:1,2                          | 118                    | 23.7     | Abony (I 1,4,[5],12,27:b:e,n,x) (3), Typhimurium (I 1,4,[5],12:i:1,2) (3), Derby (I 1,4,[5],12:f,g:[1,2]) (2), Schleissheim (I 4,12,27:b:-) (2), Saintpaul (I 1,4,[5],12:e,h:1,2) (2), <i>Salmonella</i> subsp. (2), Uppsala (I 1,4,12,27:b:1,7) (2), Athinai (I 6,7:i:e,n,z <sub>15</sub> ), Agona (I 1,4,[5],12:f,g,s:[1,2]), Brandenburg (I 4,[5],12:l:v:e,n,z <sub>15</sub> ), Fortune (I 1,4,12,27:z <sub>10</sub> :z <sub>6</sub> ), Hato (I 1,4,[5],12,g,m,s:-), Indiana (I 1,4,12:z:1,7), Lagos (I 1,4,[5],12:i:1,5), Onarimon (I 1,9,12:b:1,2) Sandiego (I 4,[5],12:e,h,e,n,z <sub>15</sub> ), Wagania (I 1,4,12,27:b:e,n,z <sub>15</sub> ), II Chartres (I 4,12:-:e,n,x:1,2,7), Abony (I 1,4,12,27:b:[e,n,x]), <i>Salmonella</i> subsp. II Enteritidis (I 1,9,12:[f]g,m,[p]h:[1,7]) (2), Javiana (I 1,9,12:l:z <sub>6</sub> :1,5) (2), Kapemba (I 9,12:l:v:1,7) (2), Lawndale (I 1,9,12:z:1,5) (2), Dublin (I 1,9,12:[Vi]:g,p:-), Goettingen (I 9,12:l:v:e,n,z <sub>15</sub> ), London (I 3,10,[15]:l:v:1,6), Irumu (I 6,7:l:v:1,5), Italiana <sup>b</sup> Itami (I 9,12:l:z <sub>13</sub> :1,5), Paratyphi C (I 6,7,[Vi]:c:1,5), Sarajane (I 1,4,[5],12,27:d:e,n,x), Victoria (I 1,9,12:l:w:1,5) |
| 2003 | 4.4     | Panama                  | 9,12:l,v:1,5                            | 120                    | 14.2     | Aarhus (I 18;z <sub>4</sub> z <sub>23</sub> :z <sub>64</sub> ) (9), Bouso (I 1,6,14,25:z <sub>4</sub> z <sub>23</sub> :e,n,z <sub>15</sub> ) (5), <i>Salmonella</i> subsp. III (3), <i>Salmonella</i> subsp. IV (3), Arapahoe (I 6,14;z <sub>4</sub> z <sub>23</sub> :1,5), Blukwa (I 6,14,18:z <sub>4</sub> z <sub>24</sub> :-), Chailey (I 6,8;z <sub>4</sub> z <sub>23</sub> [e,n,z <sub>15</sub> ]), Chichiri (I 6,14,24:z <sub>4</sub> z <sub>24</sub> :-), Corvallis (I 8,20:z <sub>4</sub> z <sub>23</sub> [z <sub>4</sub> ]), Memphis (I 18:k:1,5), Obogu (I 6,7;z <sub>4</sub> z <sub>23</sub> :1,5), Siegburg <sup>b</sup> Tallahassee (I 6,8;z <sub>4</sub> z <sub>23</sub> :-), Usumbura (I 6,14,18:d:1,7), Virchow (I 6,7,14:r:1,2), I 6,7;z <sub>4</sub> z <sub>23</sub> :-, <i>Salmonella</i> subsp. II  |
| 2003 | 4.6     | Havana                  | 13,23:f,g:-                             | 96                     | 19.8     | Raus (I 13,22,f,g:e,n,x) (6), Okatie (I 13,23:g:[s]:t:-) (2), Afula (I 6,7,f,g:t:e,n,x), Agbeni (I 1,13,23:g,m,[s]:[i]:-) Berta (I 1,9,12:[f]g,[i]:-), Bron (I 13,23:g,m:[e,n,z <sub>15</sub> ]) Chagoua (I 1,13,23:a:1,5), NewYork (I 13,22:g,s:-), Poona (I 1,13,22:z:1,6[z <sub>4</sub> ]), Rissen (I 6,7,14:f,g:-), Tees (I 16:f,g:-), Tschangu (I 1,13,23:e,h:1,5), Worthington (I 1,13,23:z:l,w)  |
| 2003 | 4.7     | Vinohrady               | 28:m,t:-                                | 75                     | 29.3     | Abadina (I 28:g,m:[e,n,z <sub>15</sub> ]) (4), Morillons (I 28:m,t:1,6) (4), <i>Salmonella</i> subsp. II (3), Croft (I 28:g,m,s:[e,n,z <sub>15</sub> ]) (2), Hatfield (I 28:d:1,6), Nitra (I 2,12:g,m:-), Othmarschen (I 6,7,14:g,m,[i]:-), Panama (I 1,9,12:l:v:1,5), Pomona (I 28:y:1,7[z <sub>6</sub> ]), Southbank (I 3,10,[15]:[15,34]:m,t:[1,6]) Techimani (I 28:c:z <sub>6</sub> ), Tennessee (I 6,7,14:z <sub>20</sub> :[1,2,7]), I 28,9,[m]s:[i]:1,5   |
| 2003 | 4.8     | Singapore               | 6,7:k:enx                               | 113                    | 15.0     | Thompson (I 6,7,14:k:1,5) (5), Escanaba (I 6,7:k:e,n,z <sub>15</sub> ) (2), <i>Salmonella</i> subsp. II (3), Braenderup (I 6,7,14:e,h:e,n,z <sub>15</sub> ), Kastrup (I 6,7:e,n,z <sub>15</sub> :1,6) Ljubljana (I 4,12,27:k:e,n,x), Mbandaka (I 6,7,14:z <sub>10</sub> :e,n,z <sub>15</sub> ), Norwich (I 6,7:e,h:1,6), Paratyphi C (I 6,7,[Vi]:c:1,5), Rissen (I 6,7,14:f,g:-)  |
| 2002 | 3.1     | Virchow                 | 6,7:r:1,2                               | 93                     | 18.3     | Infantis (I 6,7,14:r:1,5) (9), Colindale (I 6,7:r:1,7) (5), Galiema (I 6,7,14:k:1,2), Senegal (I 11:r:1,5), Thompson (I 6,7,14:k:1,5)   |

Continued on following page



TABLE 1—Continued

| Yr   | WHO no. | Correct serotype   | Correct formula          | No. of labs serotyping | % Errors | Deviating serotype (formula) (no. of labs) <sup>a</sup>  |
|------|---------|--------------------|--------------------------|------------------------|----------|--|
| 2002 | 3.2     | Derby              | 4,12:f,g:–               | 91                     | 7.1      | Agona (I 1,4,[5],12:f,g,s:[1,2]) (3), Essen (I 4,12:g,m:–), Fyris (I 4,[5],12:l,v:1,2), Kiel (I 1,2,12:sp:–)   |
| 2002 | 3.3     | Weltevreden        | 3,10:r:z <sub>6</sub>    | 91                     | 7.7      | Elisabethville (I 3,10:r:1,7) (3), Seegefeld (I 3,10:r:i:1,2), Ughelli (I 3,10:r:1,5), Weltevreden (I 3,10,[15]:r:z <sub>6</sub> ), Wilmington (I 3,10:b:z <sub>6</sub> )  |
| 2002 | 3.4     | Senftenberg        | 1,3,19:g,t:–             | 89                     | 17.1     | Westhampton (I 3,10,[15],[15,34]:g,s,t:–) (6), Rideau (I 1,3,19:f,g:–) (2), Catanzaro (I 6,14:g,s,t:–), Kingston (I 1,4,[5],12,27:g,s,t:[1,2]), Maiduguri (I 1,3,19:f,g,t:e,n,z <sub>15</sub> ), Suberu (I 3,10:g,m:–), <i>Salmonella</i> spp.   |
| 2002 | 3.5     | Typhimurium var. 5 | 4,12:i:1,2               | 96                     | 4.3      | Agona (I 1,4,[5],12:f,g,s:[1,2]), Choloraesus (I 6,7:c:1,5), Kingston (I 1,4,[5],12,27:g,s,t:[1,2]), Lagos (I 1,4,[5],12:i:1,5)  |
| 2002 | 3.6     | Manhattan          | 6,8:d:1,5                | 90                     | 15.4     | Dunkwa (I 6,8:d:1,7) (2), Isangi (I 6,7,14:d:1,5) (2), Muenchen (I 6,8:d:1,2:[z <sub>15</sub> ]) (2), Blockley (I 6,8:k:1,5), Bovismorbificans (I 6,8,20:r:[i]:1,5), Duesseldorf (I 6,8:z <sub>4</sub> ,z <sub>24</sub> :–), Kottbus (I 6,8:e,h:1,5), Newport (I 6,8,20:e,h:1,2:[z <sub>67</sub> ]), Yovokome (I 8,20:d:1,5) <sup>f</sup>  |
| 2002 | 3.7     | Enteritidis        | 9,12:g,m:–               | 95                     | 9.2      | Dublin (I 1,9,12:[Vi]:g,p:–) (2), Essen (I 4,12:g,m:–), Kapemba (I 9,12:l,v:1,7), Nitra (I 2,12:g,m:–), Seftenberg (I 1,3,19:g[s],t:–), Typhimurium (I 1,4,[5],12:i:1,2), <i>Salmonella</i> spp.   |
| 2002 | 3.8     | Bovismorbificans   | 6,8:r:1,5                | 91                     | 4.4      | Hindmarsh (I 8,20:r:1,5) <sup>g</sup> (4), Infantis (I 6,7,14:r:1,5), Hidalgo (I 6,8:r:[i]:e,n,z <sub>15</sub> ), Kentucky (I 8,20:i:z <sub>6</sub> ) Tallahassee (I 6,8:z <sub>4</sub> ,z <sub>32</sub> :–)   |
| 2001 | 2.1     | Agona              | 4,12:f,g,s:–             | 80                     | 18.8     | Derby (I 1,4,[5],12:f,g,s:[1,2]) (7), <i>Salmonella</i> spp. (4), Kingston (I 1,4,[5],12,27:g,s,t:[1,2]), California (I 4,12:g,m,t:–), Enteritidis (I 1,9,12:[f,g,m,p]:[1,7]), Essen (I 4,12:g,m:–)  |
| 2001 | 2.2     | Dublin             | 1,4,12:f,g:–             | 79                     | 34.2     | Enteritidis (I 1,9,12:[f,g,m,p]:[1,7]) (14), Rostock (I 1,9,12:g,p,u:–) (3), Agona (I 1,4,[5],12:f,g,s:[1,2]) (2), <i>Salmonella</i> spp. (2), Derby (I 1,4,[5],12:f,g:[1,2]), Moscow (I 1,9,12:g,q:–), Regent (I 3,10:f,g[s]:[1,6]), Togo (I 4,12:l,w:1,6), Typhi (I 9,12:[Vi]:d:–), II Neasden <sup>b</sup>  |
| 2001 | 2.3     | Infantis           | 6,7:r:1,5                | 83                     | 13.3     | Virchow (I 6,7,14:r:1,2) (3), <i>Salmonella</i> spp. (3), Colindale (I 6,7:r:1,7), Lomita (I 6,7:e,h:1,5), Oritamerin (I 6,7:i:1,5), Thompson (I 6,7,14:k:1,5), Typhimurium (I 1,4,[5],12:i:1,2)   |
| 2001 | 2.4     | Kottbus            | 6,8:eh:1,5               | 78                     | 38.0     | Newport (I 6,8,20:e,h:1,2:[z <sub>67</sub> ]) (12), Tshiongwé (I 6,8:e,h:e,n,z <sub>15</sub> ) (11), <i>Salmonella</i> spp. (4), Ferruch (I 8:e,h:1,5) <sup>b</sup> (3), Hidalgo (I 6,8:r:[i]:e,n,z <sub>15</sub> ), Kalumburu (I 6,8:z:e,n,z <sub>15</sub> ), Stourbridge (I 6,8:b:1,6)   |
| 2001 | 2.5     | Typhimurium        | 4,12:i:1,2               | 80                     | 9.6      | <i>Salmonella</i> spp. (2), Gloucester (I 1,4,12,27:i:l,w), Kiel (I 1,2,12:g,p:–), Lagos (I 1,4,[5],12:i:1,5), Paratyphi A (I 1,2,12:a:[1,5]), Sandiego (I 4,[5],12:e,h:e,n,z <sub>15</sub> )  |
| 2001 | 2.6     | Newport            | 6,8:eh:1,2               | 79                     | 17.7     | Tshiongwé (I 6,8:e,h:e,n,z <sub>15</sub> ) (8), Bardo (I 8:e,h:1,2) <sup>f</sup> (1), <i>Salmonella</i> spp. (2), Kottbus (I 6,8:e,h:1,5), Paratyphi B (I 1,4,[5],12:b:1,2), Rechovot (I 8,20:e,h:z <sub>6</sub> ), Saintpaul (I 1,4,[5],12:e,h:1,2)   |
| 2001 | 2.7     | Hadar              | 6,8:z <sub>10</sub> :enx | 76                     | 23.7     | Tshiongwé (I 6,8:e,h:e,n,z <sub>15</sub> ) (8), <i>Salmonella</i> subsp. (4), Istanbul (I 8:z <sub>10</sub> :e,n,x) <sup>f</sup> (1), Chailey (I 6,8:z <sub>4</sub> ,z <sub>23</sub> :e,n,z <sub>15</sub> ), Hadar (I 6,8:z <sub>10</sub> :e,n,x), Hidalgo (I 6,8:r:[i]:e,n,z <sub>15</sub> ), Mapo (I 6,8:z <sub>10</sub> :1,5), Quiniela (I 6,8:c:e,n,z <sub>15</sub> ), Rechovot (I 8,20:e,h:z <sub>6</sub> ) |
| 2001 | 2.8     | Enteritidis        | 9,12:g,m:–               | 76                     | 15.8     | <i>Salmonella</i> subsp. (3), Dublin (I 1,9,12:[Vi]:g,p:–) (2), Blegdam (I 9,12:g,m,q:–), Berta (I 1,9,12:[f,g,t]:–), Kottbus (I 6,8:e,h:1,5), Newport (I 6,8,20:e,h:1,2:[z <sub>67</sub> ]), Seremban (I 9,12:i:1,5), Typhimurium (I 1,4,[5],12:i:1,2), II Kuilsrivier <sup>b</sup>   |
| 2000 | 1.1     | Typhimurium        | 4,5,12:i:1,2             | 36                     | 8.3      | Farsta (I 4,12:i:e,n,x), Lagos (I 1,4,[5],12:i:1,5), Tumodu (I 1,4,12:i:z <sub>6</sub> )   |
| 2000 | 1.2     | Typhimurium        | 1,4,5,12:i:1,2           | 36                     | 11.1     | Gloucester (I 1,4,12,27:i:l,w) (2), Lagos (I 1,4,[5],12:i:1,5), Saintpaul (I 1,4,[5],12:e,h:1,2)   |
| 2000 | 1.3     | Bredeney           | 1,4,12:l,v:1,7           | 37                     | 29.7     | Typhimurium (I 1,4,[5],12:i:1,2) (3), Archaeoleta (I 4,[5],12:a:1,7) (2), Fyris (I 4,[5],12:l,v:1,2) (2), Derby (I 1,4,[5],12:f,g:[1,2]), Gloucester (I 1,4,12,27:i:l,w), Paratyphi B (I 1,4,[5],12:b:1,2), (–):eh:1,5 <sup>d</sup>  |
| 2000 | 1.4     | Newport            | 6,8:eh:1,2               | 36                     | 22.2     | Tshiongwé (I 6,8:e,h:e,n,z <sub>15</sub> ) (3), Bardo (I 8:e,h:1,2), <sup>b</sup> Braenderup (I 6,7,14:e,h:e,n,z <sub>15</sub> ), Kottbus (I 6,8:e,h:1,5), Larochelle (I 6,7:e,h:1,2), I (–):eh:1,2, <sup>b</sup> I 6,7:eh:– <sup>b</sup>  |
| 2000 | 1.5     | Saintpaul          | 1,4,12:eh:1,2            | 36                     | 30.6     | Chester (I 1,4,[5],12:e,h:e,n,x) (3), Kaapstad (I 4,12:e,h:1,7) (2), Sandiego (I 4,[5],12:e,h:e,n,z <sub>15</sub> ) (2), Kisangani (I 1,4,[5],12:a:1,2), Saintpaul (I 1,4,[5],12:e,h:1,2), Tsevie (I 4,12:i:e,n,z <sub>15</sub> ), I 4:e,h:e,n <sup>b</sup>  |
| 2000 | 1.6     | Schwarzengrund     | 1,4,12,27:d:1,7          | 36                     | 33.3     | Stanley (I 1,4,[5],12,27:d:1,2) (3), Schwarzengrund (I 1,4,12,27:d:1,7) (2), Canada (I 4,12,27:b:1,6), Derby (I 1,4,[5],12:f,g:[1,2]), Eppendorf (I 1,4,12,27:d:1,5), Gloucester (I 1,4,12,27:i:l,w), Grumpensis (I 13,23:d:1,7), Cairo, <sup>b</sup> I 4:d:– <sup>b</sup>   |
| 2000 | 1.7     | Heidelberg         | 1,4,12:r:1,2             | 35                     | 31.4     | Typhimurium (I 1,4,[5],12:i:1,2) (2), Derby (I 1,4,[5],12:f,g:[1,2]), Kisangani (I 1,4,[5],12:a:1,2), Kottbus (I 6,8:e,h:1,5), Paratyphi B (I 1,4,[5],12:b:1,2), Remo (I 1,4,12,27:r:1,7), Saintpaul (I 1,4,[5],12:e,h:1,2), Schwarzengrund (I 1,4,12,27:d:1,7), Stanley (I 1,4,[5],12,27:d:1,2), I (–):eh:1,2 <sup>b</sup>  |
| 2000 | 1.8     | Enteritidis        | 9,12:g,m:–               | 37                     | 10.8     | Berta (I 1,9,12:[f,g,t]:–), Blegdam (I 9,12:g,m,q:–), Bournemouth (I 9,12:e,h:1,2), Ndolo (I 1,9,12:d:1,5)   |

<sup>a</sup> Underlining indicates somatic factors determined by phage conversion (present only when the culture was lysogenized by the corresponding converting phage).

<sup>b</sup> Serotype submitted with no official name or antigenic formula.

<sup>c</sup> Not considered an error, since the serovar is subject to colonial form variation by the minor O antigen (O:6<sub>1</sub>).

Typhimurium that arose following loss of the phase two flagellin gene *fljB* (2).

Incorrect serovar identification was often caused by incorrect detection of the phase two flagellar antigen. In many

instances, erroneous serotyping results differed from the expected serovar only by the phase two flagellar antigen (Table 1). Discrimination within the H antigen complexes (E, G, and L) also accounted for a significant number of errors.

TABLE 2. Numbers of laboratories producing deviating results per year and region<sup>a</sup>

| Region               | Yr   | No. of laboratories | No. of strains serotyped | % of strains correctly serotyped | Countries participating in any iteration  |
|----------------------|------|---------------------|--------------------------|----------------------------------|---|
| Africa               | 2001 | 6                   | 37                       | 73.0                             | Botswana, Cameroon, Central African Republic, Democratic Republic of Congo, Ivory Coast, Mauritania, Madagascar, Mauritius, Morocco, Senegal, South Africa, Sudan, Tunisia, Uganda  |
|                      | 2002 | 9                   | 62                       | 87.1                             |   |
|                      | 2003 | 11                  | 70                       | 71.4                             |   |
|                      | 2004 | 9                   | 51                       | 62.7                             |   |
|                      | 2006 | 16                  | 95                       | 71.6                             |   |
| Asia and Middle East | 2007 | 11                  | 73                       | 80.8                             | Egypt, India, Israel, Jordan, Kuwait, Lebanon, Oman, Saudi Arabia, Syria  |
|                      | 2001 | 10                  | 60                       | 50.0                             |   |
|                      | 2002 | 5                   | 30                       | 83.3                             |   |
|                      | 2003 | 5                   | 35                       | 54.3                             |   |
|                      | 2004 | 5                   | 33                       | 54.5                             |   |
| Caribbean            | 2006 | 5                   | 35                       | 74.3                             | Barbados, Guatemala, Honduras, Jamaica, Suriname, Trinidad and Tobago   |
|                      | 2007 | 5                   | 40                       | 55.0                             |   |
|                      | 2001 | 0                   | 0                        | 0                                |   |
|                      | 2002 | 0                   | 0                        | 0                                |   |
|                      | 2003 | 3                   | 18                       | 61.1                             |   |
| China                | 2004 | 2                   | 8                        | 87.5                             | China   |
|                      | 2006 | 3                   | 14                       | 78.6                             |   |
|                      | 2007 | 2                   | 9                        | 77.8                             |   |
|                      | 2001 | 4                   | 32                       | 96.9                             |   |
|                      | 2002 | 3                   | 24                       | 100.0                            |   |
| Europe               | 2003 | 8                   | 60                       | 75.0                             | Albania, Bosnia and Herzegovina, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Macedonia, Malta, Republic of Moldova, The Netherlands, Norway, Poland, Romania, Serbia and Montenegro, Slovak Republic, Slovenia, Spain, Turkey, United Kingdom |
|                      | 2004 | 7                   | 46                       | 78.3                             |   |
|                      | 2006 | 6                   | 48                       | 85.4                             |   |
|                      | 2007 | 10                  | 80                       | 91.3                             |   |
|                      | 2001 | 43                  | 323                      | 80.5                             |   |
| North America        | 2002 | 50                  | 384                      | 90.0                             | Canada, United States   |
|                      | 2003 | 60                  | 401                      | 84.8                             |   |
|                      | 2004 | 57                  | 392                      | 84.7                             |   |
|                      | 2006 | 52                  | 403                      | 86.4                             |   |
|                      | 2007 | 54                  | 415                      | 89.4                             |   |
| Oceania              | 2001 | 4                   | 32                       | 87.5                             | Australia, New Caledonia, New Zealand, Papua New Guinea   |
|                      | 2002 | 2                   | 16                       | 100.0                            |   |
|                      | 2003 | 6                   | 41                       | 95.1                             |   |
|                      | 2004 | 8                   | 55                       | 81.8                             |   |
|                      | 2006 | 10                  | 80                       | 96.3                             |   |
| Russia               | 2007 | 12                  | 94                       | 97.9                             | Belarus, Georgia, Russia, Ukraine.  |
|                      | 2001 | 4                   | 30                       | 100.0                            |   |
|                      | 2002 | 6                   | 43                       | 93.0                             |   |
|                      | 2003 | 6                   | 46                       | 93.5                             |   |
|                      | 2004 | 5                   | 38                       | 97.4                             |   |
| Latin America        | 2006 | 5                   | 37                       | 94.6                             | Argentina, Bolivia, Brazil, Chile, Colombia, Costa Rica, Cuba, Ecuador, Mexico, Panama, Paraguay, Peru, Uruguay, Venezuela  |
|                      | 2007 | 4                   | 32                       | 100.0                            |   |
|                      | 2001 | 1                   | 8                        | 12.5                             |   |
|                      | 2002 | 1                   | 8                        | 62.5                             |   |
|                      | 2003 | 1                   | 7                        | 14.3                             |   |
| Southeast Asia       | 2004 | 4                   | 26                       | 69.2                             | Cambodia, Indonesia, Japan, Malaysia, Philippines, Singapore, South Korea, Sri Lanka, Taiwan, Thailand, Vietnam   |
|                      | 2006 | 5                   | 40                       | 80.0                             |   |
|                      | 2007 | 8                   | 51                       | 80.4                             |   |
|                      | 2001 | 11                  | 78                       | 57.7                             |   |
|                      | 2002 | 11                  | 82                       | 87.8                             |   |
|                      | 2003 | 13                  | 83                       | 75.9                             |   |
|                      | 2004 | 15                  | 88                       | 79.5                             |   |
|                      | 2006 | 13                  | 84                       | 84.5                             |   |
|                      | 2007 | 15                  | 107                      | 88.8                             |   |
|                      | 2001 | 15                  | 113                      | 54.0                             |   |
|                      | 2002 | 12                  | 90                       | 92.2                             |   |
|                      | 2003 | 15                  | 100                      | 81.0                             |   |
|                      | 2004 | 17                  | 130                      | 81.5                             |   |
|                      | 2006 | 15                  | 117                      | 84.6                             |   |
|                      | 2007 | 19                  | 140                      | 91.4                             |   |

<sup>a</sup> No data are available for 2000.

The overall percentage of correctly serotyped isolates differed by region. The highest numbers of errors were observed in Central Asia and the Middle East, where little improvement was seen during the seven iterations (50% in 2001 and 55% in

2007). The number of participants in the Oceanic region has been consistent ( $n = 4$ ), and all four laboratories correctly serotyped all eight strains in both 2001 and 2007. In Southeast Asia, the percentage of correctly serotyped isolates increased

TABLE 3. Numbers (and percentages) of participating laboratories that correctly serotyped all eight *Salmonella* isolates in EQAS (2000 to 2007) and total number of isolates correctly serotyped per iteration

| Yr      | No. (%) of laboratories per iteration that correctly serotyped all eight isolates <sup>a</sup> | Total no. (%) of isolates correctly serotyped per iteration <sup>b</sup> |
|---------|--|--|
| 2000    | 34 (92)  | 165 (76)   |
| 2001    | 79 (82)  | 513 (72)   |
| 2002    | 80 (81)  | 668 (91)   |
| 2003    | 69 (54)  | 692 (80)   |
| 2004    | 78 (61)  | 701 (81)   |
| 2006    | 105 (81)   | 808 (85)   |
| 2007    | 109 (78)   | 920 (88)   |
| Overall | 554 (76)   | 4,467 (82)   |

<sup>a</sup> Does not include laboratories that serotyped fewer than eight isolates.

<sup>b</sup> Includes all correctly serotyped isolates, disregarding the fact that some participants attempted to serotype fewer than eight test strains.

significantly ( $P = 0.01$ ), from 54% in 2001 to 92% in 2002, and remained consistently over 80% through 2007. In Latin America, performance increased significantly ( $P < 0.01$ ), from 58% in 2001 to 89% in 2007. Europe had the highest number of participating laboratories, and the percentage of correctly serotyped isolates ranged from 81% in 2001, when 43 laboratories participated, to 89% ( $P < 0.01$ ) in the 2007 iteration, when 54 laboratories participated (Table 2).

## DISCUSSION

Through training, reference testing services, technical support, and hosting of scientists, WHO Global Salm-Surv has been working to improve the laboratory capacity and thereby the data quality of WHO member states. The EQAS is one of the tools the program uses to assess the impact of its capacity-building efforts and to pinpoint areas for improvement.

The results from the first seven iterations of the WHO Global Salm-Surv EQAS, which to our knowledge is the largest external quality assurance program for the serotyping of *Salmonella* species, indicate that the majority of participating laboratories worldwide are capable of correctly serotyping *Salmonella* species. The number of participating laboratories increased consistently from 2000 to 2007, demonstrating an increased interest in quality assurance and an increased global capacity for *Salmonella* serotyping. However, fluctuations in the performance of some laboratories were observed. Some laboratories still do not meet the WHO Global Salm-Surv goal of performing serotyping on all eight strains with no more than one incorrect result. Efforts at building laboratory capacity for serotyping should focus on those laboratories in the future and should be directed at common difficulties.

Since test strains differ from year to year, an improvement in the performance of participating laboratories can be evaluated only on the basis of the internal quality controls. The results of quality control have remained fairly consistent during the seven iterations despite a large increase in the number of participants. The selection of a common serovar (*Salmonella* serovar Enteritidis) may have biased the results: this serovar is frequently encountered, and many laboratories are proficient at its identification. Additionally, this is a monophasic serovar. We have shown that most incorrect results appear to be caused

by errors in the identification of phase two flagellar antigens. These factors might have contributed to the consistently high performance observed for the *Salmonella* serovar Enteritidis strain. Selection of a diphasic serovar may reduce this bias in the future.

Our data suggest that several factors contributed to the observed errors. Unpublished data from a needs assessment in the EQAS 2007 iteration, where 82 laboratories (56%) completed the survey, showed that nearly 1 out of 3 (30%) laboratories have limited access to high-quality antisera. Additionally, less-common serovars were included in some iterations (e.g., *Salmonella enterica* serovar Vinohrady [I 28:m,t;-]), and the same needs assessment found that 26% of laboratories had difficulty serotyping rare and unusual *Salmonella* strains. Finally, there are important regional differences in laboratory capacity. The needs assessment found that institutions in Africa, Asia and the Middle East, and Southeast Asia were more likely to report difficulty obtaining antisera, especially for serotyping unusual *Salmonella* strains, than were institutions in other regions.

The decline in the proportion of serotypes correctly identified in 2003 and 2004 was likely due to the selection of the *Salmonella* isolates (Table 3). In 2003 and 2004, laboratories needed less-common antisera in order to fully serotype all of the EQAS isolates. After 2004, only more-common serovars were included. The subsequent overall improvement in performance suggests that many laboratories have access only to commonly available antisera. WHO Global Salm-Surv has demonstrated that the predominant *Salmonella* serotypes differ between regions (3). Therefore, a broad selection of antisera for *Salmonella* surveillance is needed globally. WHO Global Salm-Surv continues to provide information to participants on where to purchase high-quality antisera and to support many laboratories with antisera for surveillance purposes.

Many of the incorrect serotyping results were due to incorrect identification of phase two flagellar antigens (Table 1). For example, common incorrect results included misidentification of *Salmonella* serovar Typhimurium (I 4,5,12:i:1,2) as *Salmonella enterica* serovar Farsta (I 4,5,12:i:e,n,x), Lagos (I 4,5,12:i:1,5), or Tumodu (I 4,5,12:i:z6). All four serovars share the same O and phase one flagellar antigens but differ in their phase two flagellar antigens.

Colonial form variation (the variable expression of minor antigens by different single-colony picks from the same strain) may occur with the expression of the O:6<sub>1</sub> antigen by some serogroup C<sub>2</sub> serovars (10). Therefore, although the current Kauffmann-White scheme regards O:6,8 and O:8 serovar pairs, such as *Salmonella* serovars Newport (I 6,8:e,h:1,2) and Bardo (I 8:e,h:1,2), as distinct serovars, we allowed for colonial form variations. We considered correct identifications for *Salmonella* serovars Newport, Kottbus, Hadar, Manhattan, and Bovismorbificans on the basis of the serogroup alone and accepted as correct for those serovars, respectively, *Salmonella* serovars Bardo, Ferruch, Istanbul, Yovokome, and Hindmarsh.

The results from the WHO Global Salm-Surv EQAS also demonstrate important regional differences in the serotyping results for *Salmonella* species. Particular efforts should focus on Central Asia and the Middle East, but also on Africa, Russia, and the Caribbean, where a large proportion of the

laboratories do not correctly serotype many of the strains. Addressing the regional differences will involve additional training courses in selected regions.

WHO Global Salm-Surv is a platform that can assist WHO member states to strengthen their core public health capacities under the International Health Regulations (IHR, 2005) for disease surveillance and response, which will in turn strengthen international public health security. WHO Global Salm-Surv promotes intersectoral collaboration among human health, veterinary, and food-related disciplines in food safety and other issues that arise at the human-animal interface. As the program continues to expand, it increasingly addresses regional training and support needs.

**Conclusion.** This study showed that there is a continuing need to improve *Salmonella* serotyping and that this need appears to be greater in specific regions. Detection of the phase two flagellar antigen is one of the more profound barriers to obtaining a satisfactory serotyping result. Future training efforts should be aimed at enhancing the ability to characterize the phase two flagellar antigen and disseminating information on where to purchase high-quality antisera.

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**Global monitoring of *Salmonella* serovar distribution based on quality assured data from the WHO Global Salm-Surv Country Data Bank; 2001 – 2007.**

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Global monitoring of *Salmonella* serovar distribution based on quality assured data from the WHO Global Foodborne Infections Network Country Data Bank; 2001 – 2007.

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## **ABSTRACT (FPD)**

We summarised the global distribution of the 20 most commonly identified *Salmonella* serovars recovered from human clinical samples by extracting global data from selected data sets. The data for this summary was obtained from the Global Foodborne Infections Network (GFN) (formerly known as WHO Global Salm-Surv) Country Data Bank (CDB) and was based on quality-assured national or institutional data from 37 countries which passed the assurance threshold of the GFN External Quality Assurance System (EQAS). The purpose of the summary was to uncover regional and global trends in the distribution of *Salmonella* serovars. The data presented in this study could provide guidance which can be used for targeted interventions and control programmes in specific reservoirs from which these *Salmonella* serovars originate.

We found considerable regional variation among the most commonly reported serovars. However, countries within the same region typically reported similar serovars. A decreasing tendency to isolate and serotype *Salmonella* in the countries included in this study were also observed. A few serovars predominate worldwide, but were present with different frequencies in different regions. Interestingly, we observed that globally the relative percentages of *S. Enteritidis* and *S. Typhimurium* are decreasing, while other serovars such as *S. Typhi*, *S. Infantis*, *S. Hadar*, *S. Newport*, *S. Virchow*, *S. Agona* and other serovars are increasing. Given the global nature of travel and food trade, these findings suggests that further control measures have to be developed and implemented at a global scale.



## INTRODUCTION

*Salmonella enterica* is a common cause of human gastroenteritis and bacteremia worldwide. It is estimated that in the United States alone there are 1.4 million non-typhoidal *Salmonella* infections, resulting in 168,000 physician visits, 15,000 hospitalizations and 580 deaths (Voetsch *et al.*, 2004). A wide variety of animals, particularly food animals, have been identified as reservoirs for non-typhoidal *Salmonella* (Coyle *et al.*, 1988; Humphrey *et al.*, 1988; Humphrey *et al.*, 2000).

Although more than 2,500 serovars of *Salmonella enterica* have been identified to date, most human infections are caused by a limited number of serovars. In most developed countries, *Salmonella enterica* serovars Typhimurium and Enteritidis are the most commonly reported causes of human salmonellosis, however other serovars appear to be more prevalent in specific regions (Humphrey *et al.*, 2000; Henrikstad *et al.*, 2002; Olsen *et al.*, 2001; Bangtrakulnonth *et al.*, 2004; Galanis *et al.*, 2006). Shifts in the proportion of specific strain types and serovars in human and animal populations can be due to their introduction through international travel, human migration, and globalization of food and livestock trade. Thus, the occurrence of *Salmonella* in one country presents a potential problem for other countries. To minimize the burden of this pathogen; it is necessary to monitor *Salmonella* serovar distribution in all countries, implement *Salmonella* control measures throughout the food production chain, and monitor the effectiveness of the control measures. Most scientific studies on the control of *Salmonella* have been directed towards *S. Enteritidis* and *S. Typhimurium*. While is expected that these control measures are also effective against other serovars, this concept has not been sufficiently evaluated.

In January 2000, the World Health Organization (WHO) launched the WHO Global Salm-Surv programme (WHO GSS), a global effort to build capacity to detect, control and prevent foodborne and other enteric infections from farm to table. Though originally focusing on *Salmonella* diagnostics and epidemiology, the programme has evolved into a capacity-building platform that accommodates a variety of foodborne and other enteric pathogens and diseases of importance in the various regions. In order to reflect this broader scope and application, the WHO GSS network recently changed name to "Global Foodborne Infections Network (GFN)". Enhancing worldwide laboratory capacity for the serotyping of *Salmonella* is still a key objective of GFN and this objective is facilitated by bench top training at international courses and workshops.

Additional objectives of the GFN program include enhancing laboratory based surveillance networks and strengthening laboratory quality management systems. The External Quality Assurance System (EQAS) and the Internet-based Country Data Bank (CDB) are two key GFN programs which are used to help accomplish these objectives. To ascertain the performance of participating laboratories and thereby promote enhanced laboratory-based surveillance, an annual External Quality Assurance System (EQAS) was established in 2000 as a part of the GFN programme (Petersen *et al.*, 2002; Hendriksen *et al.*, 2008; Hendriksen *et al.*, 2009). The Internet-based Country Data Bank (CDB), is an online database to which member countries are encouraged to annually upload data on their 15 most commonly identified *Salmonella* serovars (<http://thor.dfvf.dk/gss>). Anyone with internet access may then query the databank and view data on serovar prevalence by country and region.

In this paper, we summarised the distribution of *Salmonella* serovars for selected countries, based on extracted global data from selected data sets data in the CDB. The purpose of this study was to uncover trends and patterns in *Salmonella* serovar prevalence during the period from 2001 to 2007. This analysis and summary was based on data from laboratories in countries which passed the EQAS quality assurance threshold (eight isolates tested with a maximum of one deviation from the expected serotyping result) established by the steering committee of the GFN

## METHODS

### *External Quality Assurance System*

The EQAS is described in detail elsewhere (Petersen et al., 2002; Hendriksen *et al.*, 2009). In brief, between 140 and 180 participating laboratories receive eight isolates of *Salmonella* on an annual basis. The laboratories are requested to serotype the strains and submit their results through a password protected database to the WHO Collaborating Centre for Antimicrobial Resistance in Foodborne Pathogens, Copenhagen, Denmark (the coordinating laboratory). The participants then instantly obtain an evaluation report with suggestions for corrective actions. The data are stored centrally, summarized and an annual report is published online (<http://www.antimicrobialresistance.dk/233-169-215-eqas.htm> or [http://www.who.int/salmsurv/activities/GSS\\_EQAS/en/](http://www.who.int/salmsurv/activities/GSS_EQAS/en/)).

### *Country data bank*

The CDB is a web-enabled Oracle database. Only authorised members may enter data into the CDB and data entry is password protected. However, the CDB may be viewed and queried by the general public. Each year, a designated national laboratory representative enters either summarised institutional or national data into the CDB. The entered data sets consist of the

number of *Salmonella* isolated and serotyped from human, animal, food, environmental, and/or feed sources and the 15 most frequently identified serovars from each of these reservoirs. Since the inception of the programme in 2000, the number of GFN memberships registered in the CDB has increased steadily and in 2009 to a total of over 1,200 members in 158 countries. Throughout this period, 157 GFN “member institution representatives” have been designated. These member institution representatives are allowing to upload national data on the top 15 *Salmonella* serovars by reservoirs. An additional 445 “general institutional representatives” were given rights to upload institutional data to a separate part of the CDB. In total, 987 national data sets and 177 institutional datasets have been uploaded into the CDB.

Member lists of the GFN EQAS and the CDB were compared in a central Oracle database and a list of institutional and national members participating in both programme components was obtained. Only data from members who had uploaded summarised data of human origin into the CDB and met the quality assurance threshold in one or more iterations of the EQAS were included in the analysis. Non-human data (i.e. animal, food, or environmental) was omitted from the study due to the small number of members who both uploaded data from non-human sources and met the quality assurance threshold. Member data (number of *Salmonella* isolated and serotyped as well as the top 15 most common serovars from human isolates), originating from uneven years from 2001 to 2007, was listed in the file by world macro region (i.e. Europe, Asia, Oceania, Africa, North America and Latin America) and sorted by the occurrence of the serovar in countries and by year. For this study only results of the top 20 most frequent serovars per region were included (Tables 1 - 6).

#### *Descriptive and statistical analyses*

Descriptive analyses, at the national and macro regional level, of the proportion of serovars were performed, based on the data reported by the countries/institutions and the population estimates. Further descriptive analysis on the observed differences in serovar distribution was performed using classifications provided by the International Monetary Fund (IMF)

(<http://www.imf.org/external/index.htm>) and the Central Intelligence Agency (CIA)

(<https://www.cia.gov/library/publications/the-world-factbook/index.html>). These sources were used to obtain population estimates and to classify the countries as developed (Australia, Canada, Denmark, Finland, Germany, Greece, Israel, Italy, Japan, Luxembourg, Malta, Netherlands, New Zealand, Spain, United States) or developing (all other participating countries).

Cochran-Armitage trend test, which is fully described in the SAS documentation, was used to study the significance of reported trends over the years and tests with  $p$ -values  $<0.05$  were assumed as significant trends. Linear regression was used to develop trend lines of the serovars proportion within the macro regions. SAS Enterprise Guide 3.0 (SAS Institute, Cary, NC) was used to data handling and analysis and Microsoft Excel (Microsoft Corp, Redmond, WA) to plot the figures.

## RESULTS

### *Content of the dataset*

The dataset extracted from the CDB and included in this paper originated from 37 countries (Public Health laboratories) within six regions. Data from an additional 38 laboratories from 35 countries which have participated in one or more EQAS iterations was excluded from analysis because the laboratory failed to meet the quality threshold of the EQAS (18 laboratories) (i.e.

more than 1/8 deviations from the expected results), the laboratory uploaded institutional data but national data were available and included (eight laboratories) or because the laboratory did not submit data of human origin (12 laboratories). The final dataset comprised of observations from countries submitting data between one to all four years in this study (Tables 1 - 6). Between the study years, the number of countries in this subset of the CDB submitting data differed greatly, with 33 countries submitting data in 2001, 34 in 2003, 22 in 2005 and only 19 countries submitting data in 2007. The average number of isolates serotyped per 100,000 inhabitants per region in all years fluctuated from 3.5 in the Latin American region to 82.4 in the Oceania region. However, the average number of isolates serotyped per 100,000 inhabitants by all countries decreased over the years, from 9.1 in 2001 to 1.9 in 2007.

A large variation in the number of serovars was observed, at the regional level. The highest number of serovars (n=92) was reported from 17 countries in the European region. North America (the United States and Canada) reported the fewest serovars (n=22). In the Oceania region, two countries submitted data in the period (2001 – 2007) and 31 different serovars were reported. Three countries reported data from the African Region accounting for 73 different serovars. Despite reporting different serovars and different percentages, the Latin American and the Asian regions both reported nearly the same number of different serovars; 59 (eight countries) and 54 (five countries), respectively.

#### *Parameters influencing the distribution of serovars between regions and countries.*

We observed that the consistency with which serovars were reported by the countries varied both by macro-region and development status. In general, the spectrum and percentages serovars

reported by developed countries remained fairly consistent; whereas considerable year to year variability in both spectrum and percentage of serovars was observed in developing countries. For some of the most commonly reported serovars, the proportions reported by each country were plotted by region. A regression line was drawn on these plots, to visualize trends over years (Figures 2 and 3). In a following step, proportions were estimated at the country development level, grouping all the participating countries into developing and developed countries. Trends over the years were plotted, again demonstrating differences in the proportion trends among the evaluated groups (Figure 4).

#### *S. Enteritidis and S. Typhimurium*

In all regions with exception of the Oceania and North America regions, *S. Enteritidis* and *S. Typhimurium* ranked as the most common and second most common serovar, respectively. In North America and the Oceania regions *S. Typhimurium* was the most common serovar reported and *S. Enteritidis* was the second most common serovar. (Table 4 and 5).

Globally the overall proportion of these two serovars has decreased over time with *S. Enteritidis* decreasing from 44.2% to 41.5% and *S. Typhimurium* decreasing from 18.9% to 15.0% (Figure 1). This was mainly due to a significant decreasing trend ( $p<0.01$ ) in the proportion of *S. Enteritidis* in developing countries and a non-significant decreasing trend ( $p=0.16$ ) in the proportion of *S. Typhimurium* in developed countries (Figure 4). Furthermore, the trends differed between regions and countries (Figure 2 and 3). Thus, the proportions of *S. Enteritidis* presented a significant increasing trend in Africa ( $p=0.02$ ) and Oceania ( $p=0.04$ ) and a non-significant increasing trend in North America ( $p=0.13$ ). However, the proportions presented non-significant decreasing trend in Asia and Latin America and were nearly constant in Europe (Figure 2). *S.*

Typhimurium increased in South America, but decreased in all other regions, except Asia (Figure 3). However, none of these regional trends were statistically significant.

The changes were not equally distributed between countries within the same region. Thus, the increase of *S. Enteritidis* in Africa was due to a major increase of this serovar in Tunisia and the decrease of *S. Typhimurium* was due to a decrease of this serovar in Cameroon (Table 1). Similarly the decrease of *S. Enteritidis* in Asia was due to decreases in Japan and Korea (Table 2). *S. Enteritidis* increased in three countries of Latin America, but decreased in four (Table 6).

### *S. Typhi*

An increase in the proportion of *S. Typhi* in all regions began to be observed in 2003. Since 2003, the overall proportion of *S. Typhi* has increased from 0.7% to 2.2% (Figure 1). This increase was observed in both developed and developing countries, during the studied period (Figure 4).

In the Oceania region, *S. Typhi* was the eighth most common serovar reported to the CDB from New Zealand (Table 5). A similar level was observed in the North American region (Table 4). In the African region, *S. Typhi* was ranked as the fifth most common serovar. In Senegal, *S. Typhi* decreased from 7.8% to 4.9% (Table 1). On the contrary, the proportion of *S. Typhi* increased from 4.8% to 16.2% in Cameroon and was first reported in Tunisia among the top 15 in 2007 (Table 1).

All countries, with the exception of Costa Rica, Paraguay and Uruguay in the Latin American region reported *S. Typhi* among the top 15 most common serovars nationally. In Brazil and Chile, the proportion presented decreasing trends ( $p=0.07$  and  $p<0.01$  respectively), whereas an



increasing trend was reported in Colombia ( $p<0.01$ ) (Table 6). In the Asian region, *S. Typhi* was the 3<sup>rd</sup> most common serovar reported by the Republic of Korea (3<sup>rd</sup>) and the most common serovar reported by Malaysia and the Philippines (Table 2). In the European region, *S. Typhi* was in general listed only as the 35<sup>th</sup> most common serovar (Data not shown).

#### *Other non-typhoidal Salmonella serovars*

In addition to *S. Enteritidis* and *S. Typhimurium*; *Salmonella* Infantis was also among the serovars reported from all regions (Table 1 - 6). Globally, the overall proportion of *S. Infantis* increased over the years, from 1.5% to 2.2%. However no statistically significant increasing trend was detected ( $p=0.76$ ) (Figure 1). This increase was mainly associated with the developed regions (Figure 4). The serovar was ranked as the fifth most common serovar in the European region (Table 3ab).

*Salmonella* Agona was frequently observed in Asia and Latin America, ranking among the top three most common serovars. This serovar was also ranked seventh in Europe and 13<sup>th</sup> in North America (Tables 3 and 4). Overall, the proportion of this serovar increased from 0.8% to 1.5% between 2001 and 2007. This increase was observed in both develop and developing countries, and was accentuated between 2005 and 2007 (Figure 4).

A slight decrease in the overall proportion over time (2.5%-2.3%) was seen for *Salmonella* Heidelberg (Figure 1). This serovar was more common among developed countries (Figure 4). While *S. Heidelberg* ranked fourth in North America; lower frequencies were seen in Europe (9<sup>th</sup>) and Latin America (19<sup>th</sup>) (Tables 3, 4 and 6).

*Salmonella* Virchow was only reported among the top serovars in Asia, Europe and the Oceania regions. However, in these regions *S. Virchow* often represented a high proportion of the *Salmonellae* isolated in these regions (Table 2, 3 and 5). The overall proportion of this serovar has oscillated over the years (Figure 1) and, since 2005, an increase has been seen among developed countries with proportional decrease reported by developing countries (Figure 4).

High frequencies of *Salmonella* Thompson were seen in Europe and North America (Table 3 and 4). Additionally, *Salmonella* Newport has consistently been reported among the top six serovars by the North American, European, and Latin American regions. Nevertheless; the overall proportion of *S. Newport*, which increasing in the initial years of the study, decreased from 5.0% in 2005 to only 1.2% in 2007 (Figure 1). *Salmonella* Oranienburg was only observed in North and Latin America, and was reported as the 10<sup>th</sup> and 15<sup>th</sup> most common serovar, respectively (Table 4 and 6).

*Salmonella* Hadar and *Salmonella* Montevideo were reported by almost all regions. However the frequencies varied considerably. *S. Hadar* was observed among the top three serovars in Europe, but was seen at lower frequencies in other regions. In general, with the exception of a slight decrease in 2005, the overall proportion of *S. Hadar* remained relatively constant throughout the survey period (Figure 1). Finally, *Salmonella* Montevideo was more common in North- and Latin America while *Salmonella* Saintpaul was more predominant in Oceania and North America. Both serovars presented the same proportional trends over time; increases in both serovars were observed in 2003 and 2005; these increases were followed by a decrease in 2007 (Figure 1).

## DISCUSSION

This study is based on reported data from reference laboratories in 37 countries. This only represents about 20% of all WHO Member States. Despite the fact that some geographical regions are represented by a limited number of countries, we believe that the data are broad enough and of sufficient quality to describe global trends in *Salmonella* serovar distribution.

#### *Content of the data set*

For Cameroon, Malaysia, Belarus, Croatia, Germany and Malta, no national data were available in the CDB. For these countries, we have included institutional data from institutes that met the quality standard threshold of the EQAS. Institutional data from these countries was included to obtain a better coverage of countries in the regions (Table 1- 6). For some countries, national data submitted by the national reference laboratory might also include data collected from other institutes which may or may not have been participating in the EQAS programme. Consequently, the validity of these data is not entirely known, but is expected to be high, based on the skills and quality of the national reference laboratories. In general, the highlighted serovars among humans presented by Galanis et al. (2006) based on non-selected data available in the CDB between 2000 and 2002 seem to be in agreement with the data from this study submitted by laboratories that perform at or above the quality standard threshold of the EQAS. This observation suggests that the majority of the human data in the CDB could be regarded as valid and therefore be included in future analysis of aggregated data. However, as the EQAS results of individual laboratories are confidential, the general user of the CDB data cannot assess the validity of data provided by a single source. Therefore, care should be taken when interpreting results from direct comparisons between single countries, especially when these are geographically distinct and have no links through travel or trade,

We observed a decreasing number of isolates being serotyped. We do not know if this reflects a true global decrease in the occurrence of *Salmonella* spp., a global decrease in national funds available for this area of public health care or fewer countries reporting to the CDB. The CDB was designed to be a self-updating database where the designated members are responsible for populating the database. However, the submission of data to the CDB is voluntary and the task is not always automatically transferred to a successor when a designated member institution representative leaves the post.

The total number of isolates serotyped annually was converted into an incidence (i.e. the number of isolates serotyped per 100,000 inhabitants per year), to give a better comparison of the numbers between countries. There are many potential reasons why these incidence rates may differ between countries. An apparently low incidence rate of human *Salmonellosis* may be due to geographical, cultural or socio-economic factors in-country which limit the population's access to medical care; limited access to laboratory testing; or a truly low incidence of *Salmonella* in these countries. In contrast, a high incidence rates may be due to a national outbreak in a specific year or time period. The CDB does not gather this information when representatives submit data from their countries. The incidence is certainly also influenced by active surveillance programmes that are in place in several countries. However, we believe this to have a minor effect on the ranking of the serovars as long as the data is captured from a representative sample of the population. Based on our data we could not identify a particular country which was performing a better monitoring of *Salmonella* than others.

There were major differences among the most commonly isolated serovars between regions but fewer differences between countries within the same region. In addition, we observed major

differences between countries and regions in serovars detected by year and we believe that several parameters might have influenced these differences. In developed countries, the production of food animals are narrowed to the most common species / breeds and the production systems are often effective and high scale. In addition, the production stock commonly stems from highly centralized rearing facilities. This has a great potential for spread of one or more *Salmonella* serotypes to a large number of herds and flocks, as was demonstrated by the *S. Enteritidis* pandemic throughout the nineties which affected both developed and developing countries. In developing countries, the production scale is normally not intensive. In addition, developing countries in tropical regions typically raise native crops and animals which are then consumed locally. This locally produced food may harbor a greater diversity of less commonly reported serovars.

#### *S. Enteritidis and S. Typhimurium*

Consistent with other studies; our data revealed that *S. Enteritidis*, followed by *S. Typhimurium* were the most frequently isolated serovars from humans worldwide. This trend was also observed in an article describing non-selected data from the CDB in 2000 to 2002 (Galanis *et al.*, 2006). Interestingly, despite some fluctuations between countries we observed a global decrease in the proportion of *S. Enteritidis* and *S. Typhimurium*. It is still too early to determine whether this is a true and stable global trend, but it might indicate that these two serovars on a global scale are decreasing. This decrease could be explained by the intense focus worldwide on these particular serovars and the introduction of specific monitoring and control programmes.

#### *S. Typhi*

Overall, the proportion of *S. Typhi* was increasing during the study period. *S. Typhi* was by far the most commonly reported of the typhoidal serovars and present among the top 20 most common serovars in almost all of the regions, including both developing and developed countries. However, specific countries and regions seem to be much more affected by this serovar than others. In endemic countries, Typhoid fever is typically associated with poor-sanitation (Vollaard *et al.*, 2004); in developed countries, most cases of typhoid occur in travellers returning from developing countries (Steinberg *et al.*, 2004). While the epidemiology of typhoid varies by development status, these data suggests that there is a need to increase awareness and prevention measures of the typhoidal *Salmonella* in both developed and developing countries.

#### *Other non-typhoidal Salmonella serovars*

A few serovars predominate worldwide, which we believe is caused mainly by central rearing of production animals, international trade, and travel. It was not possible to test these hypothesis within the scope of these data but several publications support the observation that serovars follow trade patterns. For example, a recent outbreak in the United States was caused by *S. Saintpaul* which originated from imported jalapenos peppers from Mexico (Centers for Disease Control and Prevention (CDC)). In 2004, several countries in Europe had observed a regional outbreak caused by *S. Thompson* in rucola lettuce originating from one producer in Italy (Nygård *et al.*, 2008). Data from recent studies have identified indistinguishable PFGE patterns of *S. Schwarzengrund* from patients in Denmark, the United States and Thailand and chicken meat originating from Thailand (Aarestrup *et al.*, 2007). Similarly, identical clones of *S. Rissen* were found among Thai patients and Danish patients with a history of travel to Thailand (Hendriksen *et al.*, 2008). Source attribution data from Denmark also support that trade and international travel play a major role in the transmission of *Salmonella* (Hald *et al.* 2007). It is noteworthy that

while *S. Enteritidis* and *S. Typhimurium* decreased during the observational period, a number of other serovars such as *S. Typhi*, *S. Infantis*, and *S. Virchow* increase in relative importance. This could indicate that some of the control measures taken against *S. Enteritidis* and *S. Typhimurium* are not equally efficient against other serovars or that other sources which act as reservoirs for these serovars have become increasingly important (e.g. unconventional food animal species, wild or free-range domestic animals). Therefore, we may have to develop and implement novel control strategies for these serovars. To determine the sources towards which control programmes should be initiated, as well as to determine the impact of the programmes in place, there is a continuing need for national, regional and global monitoring.

#### *Future aspects*

The data presented in this study is the first attempt to establishing a global monitoring based on quality assured data of the occurrence of *Salmonella* infections in humans. These data provide microbiologists and epidemiologists with a unique tool to understand the epidemiology of *Salmonella* worldwide in humans. Today, we are all residents of a global village; with the increased trade of food, livestock, as well as increased travel and human migration; infectious disease is no longer confined to a single country. The data presented in this study reveal the complexity of global epidemiology, particularly as frequency and occurrence change over time in countries and regions. Nevertheless, epidemiologists may gain valuable knowledge for outbreak detection and hypothesis generation regarding the origin of the infections by knowing which serovars predominate in which regions. This information might lead the investigation to a specific country to which patients have travelled or where a certain food source originates. The data could also serve as a tool for launching targeting interventions to diminish the burden of salmonellosis caused by specific serovars. The data reveal that the developing regions may

harbour a niche to foster new emerging serovars which potentially could be critical for global food safety. This may require food safety authorities to alternative actions in implementing intervention and control programmes in reservoirs of specific predominant *Salmonella* serotypes. This could for instance be a targeted action to decrease the frequency of *S. Typhi* in a specific region or against *S. Infantis* worldwide to avoid a new pandemic serovar such as *S. Enteritidis*. Furthermore, it is necessary to encourage food and veterinary institutes to upload additional data to the CDB, which could reveal linkages between human illness and the different reservoirs of the *Salmonella* serovars.

## CONCLUSION

The outcome of this study shows large differences of the top 20 most commonly isolated serovars between regions but lesser differences between the top 15 most commonly isolated serovars between countries within the same region. Nevertheless, a few serovars are more frequent than others in many of the regions and countries. Our findings highlight the complexity of the global epidemiology of *Salmonella* and the urgent need and importance for improving monitoring data of those serovars of highest epidemiologic importance in order to avoid a new pandemic such as that caused by *S. Enteritidis*. We also advise carefully monitoring on a global level of some serovars, such as *S. Infantis*, *S. Hadar*, *S. Newport*, *S. Virchow* and *S. Agona*. In addition, the data show a decreasing tendency to isolate and serotype in the countries included this study. This is a bothersome trend as isolates and the subtype data obtained from them are the corner-stone of laboratory based surveillance systems for *Salmonella*. We encourage laboratories to work with their clinical counterparts to increase the number of samples submitted for culture and serotyping. We also stress the use of quality methods and the timely reporting of data to national surveillance networks. The effect of using quality assured data, based on the EQAS participation revealed to



be minor, but we recommend using aggregated data in contrast to data originating from single institutes. The data presented in this study could guide the development of regional and serovar specific intervention and control programme. As such, urge food and veterinary institutes to upload the data on the 15 most commonly isolated *Salmonella* serovars to the CDB. Non-human data is essential to the CDB, this data, when supplemented with human clinical data, can help to identify important reservoirs and help initiate longer term prevention and control measures.

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Table 1. Distribution of the 20 most frequently serotyped human *Salmonella* isolates from African countries in numbers and percentages.

|                 | Cameroon*  |      |      |      |      | Senegal    |      |      |      |      | Tunisia    |      |      |      |      |
|-----------------|------------|------|------|------|------|------------|------|------|------|------|------------|------|------|------|------|
|                 | 18,467,692 |      |      |      |      | 12,853,259 |      |      |      |      | 10,383,577 |      |      |      |      |
| Year            | 2001       | 2003 | 2005 | 2007 | 2007 | 2001       | 2003 | 2005 | 2007 | 2007 | 2001       | 2003 | 2005 | 2007 | 2007 |
| Total serotyped | 173        | 182  | 151  | 99   | 99   | 232        | 176  | 151  | 102  | 102  | 720        | 599  | 363  | 243  | 243  |
| Enteritidis     | 18.3       | 14.8 | 21.2 | 19.2 | 19.2 | 19.8       | 8.0  | 19.2 | 4.9  | 4.9  | 14.3       | 27.9 | 28.1 | 49.0 | 49.0 |
| Typhimurium     | 63.7       | 53.3 | 28.5 | 18.2 | 18.2 | 4.3        | 5.1  |      | 10.8 |      |            | 4.0  | 7.4  | 11.5 | 11.5 |
| Livingstone     |            |      |      |      |      |            |      |      |      |      | 21.1       | 26.7 | 5.5  |      |      |
| Corvallis       |            |      |      |      |      |            | 1.7  | 2.6  |      |      | 14.0       | 3.5  | 6.1  |      |      |
| Typhi           | 4.8        | 17.0 | 18.5 | 16.2 | 16.2 | 7.8        | 8.5  | 7.9  | 4.9  |      |            |      |      | 2.1  |      |
| Braenderup      |            |      |      | 1.0  |      |            |      |      |      |      | 13.1       |      | 3.6  |      |      |
| Anatum          |            |      |      |      |      |            |      |      |      |      | 4.3        | 3.2  | 11.0 | 4.9  |      |
| Infantis        |            | 0.5  |      |      |      |            |      |      |      |      | 5.7        |      | 10.2 | 1.6  |      |
| Kentucky        |            |      |      |      |      | 6.0        | 9.7  | 4.0  | 3.9  |      |            | 2.8  |      | 6.2  |      |
| Cerro           |            |      |      |      |      |            |      |      |      |      | 4.3        | 3.2  | 1.1  |      |      |
| Newport         |            |      |      |      |      |            |      |      |      |      | 2.5        | 2.7  | 5.2  |      |      |
| Mbandaka        | 0.7        |      |      |      |      |            |      |      | 2.0  |      | 5.3        |      | 2.8  |      |      |
| Amsterdam       |            |      |      |      |      |            |      |      |      |      |            | 3.0  | 3.3  | 6.6  |      |
| Hadar           |            | 1.6  | 1.3  | 1.0  | 1.0  | 4.3        | 1.7  | 2.6  | 2.9  |      |            | 2.5  |      |      |      |
| Tokoin          |            |      |      |      |      |            | 17.6 |      |      |      |            |      |      |      |      |
| Zanzibar        |            |      |      |      |      |            |      |      |      |      |            |      | 4.1  | 4.1  |      |
| Altona          |            |      |      |      |      |            |      |      |      |      |            |      | 4.1  | 2.1  |      |
| Muenster        |            |      |      |      |      |            | 1.1  | 2.6  |      |      |            |      | 1.9  | 1.2  |      |
| Wien            |            |      |      |      |      |            |      |      |      |      | 2.2        |      |      |      |      |
| Bredeney        |            |      |      |      |      |            | 4.0  | 5.3  |      |      |            |      |      |      |      |

\*Institutional data

Table 2. Distribution of the 20 most frequently serotyped human *Salmonella* isolates from Asian countries in numbers and percentages.

| Population      | Japan |       |       |      | R. of Korea |      |      |       | Malaysia* |      |      |       | Philippines |       |       |      | Thailand |      |      |  |
|-----------------|-------|-------|-------|------|-------------|------|------|-------|-----------|------|------|-------|-------------|-------|-------|------|----------|------|------|--|
|                 | 2001  | 2003  | 2005  | 2007 | 2001        | 2003 | 2003 | 2005  | 2007      | 2005 | 2007 | 2001  | 2003        | 2005  | 2007  | 2001 | 2003     | 2005 | 2007 |  |
| Year            | 2,864 | 2,422 | 1,529 | 963  | 1,037       | 676  | 929  | 1,396 | 859       | 203  | 172  | 4,134 | 3,426       | 3,669 | 2,720 |      |          |      |      |  |
| Total serotyped | 52.7  | 59.2  | 47.4  | 44.7 | 42.9        | 33.7 | 21.2 | 21.9  | 34.7      | 3.9  | 5.8  | 8.6   | 11.5        | 11.0  | 16.5  |      |          |      |      |  |
| Enteritidis     |       |       |       |      |             |      |      |       |           |      |      |       |             |       |       |      |          |      |      |  |
| Weltevreden     |       |       |       |      |             |      | 15.5 | 13.5  | 17.1      | 0.5  | 0.6  | 15.9  | 11.1        | 9.0   | 6.1   |      |          |      |      |  |
| Typhi           |       |       |       |      | 14.0        | 11.2 | 36.5 | 46.0  | 9.9       | 78.8 | 86.6 |       |             |       |       |      |          |      |      |  |
| Stanley         |       |       |       | 1.2  |             |      | 1.5  | 1.1   | 2.0       | 1.0  |      | 5.9   | 8.6         | 12.4  | 10.9  |      |          |      |      |  |
| Typhimurium     | 4.4   | 7.5   | 4.1   | 6.9  | 13.9        | 16.0 | 4.2  | 3.5   | 6.1       | 1.5  | 1.2  | 4.2   | 2.6         | 7.4   |       |      |          |      |      |  |
| Rissen          |       |       |       |      | 1.5         | 1.8  |      | 0.4   |           |      |      | 6.3   | 6.4         | 8.9   | 8.1   |      |          |      |      |  |
| Anatum          |       |       |       |      |             |      |      |       |           |      | 1.2  | 8.2   | 6.4         | 4.8   | 4.5   |      |          |      |      |  |
| Corvallis       | 1.7   | 0.8   |       |      |             |      | 9.3  | 6.3   | 9.1       |      |      | 8.1   | 2.5         | 2.2   | 3.6   |      |          |      |      |  |
| I 1,4,5,12:i:-  |       |       |       | 1.6  |             |      |      |       |           |      |      |       |             |       |       |      |          |      |      |  |
| Choleraesuis    |       |       |       |      |             |      |      |       |           | 3.0  | 0.6  |       | 3.4         | 4.8   | 7.8   |      |          |      |      |  |
| Panama          |       |       |       |      |             |      |      |       |           |      |      | 3.9   | 2.9         | 1.6   | 2.6   |      |          |      |      |  |
| Virchow         | 1.3   | 1.9   | 1.0   |      |             |      |      |       | 0.4       | 0.5  |      | 2.5   | 2.4         | 2.3   |       |      |          |      |      |  |
| Infantis        | 3.9   | 4.4   | 5.2   | 5.5  |             | 0.7  |      |       |           |      |      |       |             |       |       |      |          |      |      |  |
| Albany          |       |       |       |      |             |      | 0.9  | 0.8   | 0.7       |      |      | 2.4   | 2.9         | 1.7   | 1.4   |      |          |      |      |  |
| Thompson        | 5.5   | 2.2   | 4.0   | 5.9  |             |      |      |       |           |      |      |       |             |       |       |      |          |      |      |  |
| Hadar           | 2.0   |       | 1.0   |      |             |      |      |       |           |      |      | 3.3   | 2.9         |       |       |      |          |      |      |  |
| Agona           | 1.9   | 2.1   | 1.5   | 1.0  | 0.4         | 0.9  | 0.9  | 0.6   | 1.0       |      |      |       | 2.2         |       | 1.4   |      |          |      |      |  |
| Derby           |       |       | 1.3   |      |             |      |      |       |           | 0.5  |      | 2.7   | 2.0         |       | 2.6   |      |          |      |      |  |
| Saintpaul       | 3.8   | 2.6   | 2.2   | 6.0  |             |      |      |       |           |      |      |       |             |       |       |      |          |      |      |  |
| Schwarzengrund  |       |       | 0.8   |      | 3.4         | 0.7  |      |       |           |      |      | 2.4   | 1.8         |       |       |      |          |      |      |  |

\*Institutional data

Table 3a. Distribution of the 20 most frequently serotyped human *Salmonella* isolates from European countries in numbers and percentages.

| Population<br>Year    | Belarus* |       | Bulgaria |      | Croatia* |       | Denmark |       | Estonia |       | Finland |      | Germany* |      | Greece |      |       |       |       |       |       |       |      |
|-----------------------|----------|-------|----------|------|----------|-------|---------|-------|---------|-------|---------|------|----------|------|--------|------|-------|-------|-------|-------|-------|-------|------|
|                       | 2001     | 2007  | 2001     | 2007 | 2001     | 2007  | 2001    | 2007  | 2001    | 2007  | 2001    | 2007 | 2001     | 2007 | 2001   | 2007 |       |       |       |       |       |       |      |
| Total serotyped       | 126      | 1,022 | 1,027    | 641  | 500      | 2,212 | 1,646   | 1,809 | 1,113   | 2,918 | 1,712   | 1,68 | 1,554    | 304  | 184    | 2,74 | 2,178 | 4,395 | 2,244 | 1,204 | 1,561 | 1,350 | 977  |
| Enteritidis           | 41.3     | 59.6  | 82.9     | 73.2 | 69.4     | 90.1  | 92.8    | 92.7  | 80.1    | 48.9  | 43.0    | 38.2 | 36.4     | 76.6 | 81.5   | 48.2 | 44.1  | 40.8  | 41.5  | 62.8  | 77.7  | 73.2  | 63.2 |
| Typhimurium           | 30.2     | 10.8  | 8.1      | 11.5 | 18.8     | 5.1   | 1.9     | 2.0   | 8.0     | 20.2  | 26.3    | 33.4 | 22.7     | 8.6  | 4.3    | 10.5 | 13.1  | 38.0  | 36.3  | 19.4  | 10.7  | 11.2  | 13.3 |
| Hadar                 |          | 2.1   |          | 0.8  | 0.4      | 1.5   | 0.5     | 0.8   | 0.7     | 2.1   | 1.0     | 1.4  |          |      |        | 3.6  | 2.8   | 0.5   | 0.4   | 2.0   | 0.8   | 1.0   |      |
| Virchow               |          |       |          | 0.3  |          |       | 0.2     | 0.3   | 0.4     | 1.3   | 2.3     | 2.1  | 2.7      | 0.3  |        | 3.1  | 3.2   | 0.4   |       | 0.8   | 0.4   | 0.4   |      |
| Infantis              |          |       |          |      |          |       | 0.5     | 0.3   | 0.4     | 1.5   | 1.8     | 1.5  |          |      | 2.7    | 1.8  | 0.9   | 0.7   | 0.9   | 1.1   | 0.4   | 0.5   |      |
| Newport               | 0.8      |       | 0.1      |      |          |       | 1.0     | 0.4   | 1.3     | 1.2   | 1.6     | 2.3  | 1.5      |      |        | 2.2  | 2.6   |       |       | 0.4   | 0.3   | 1.1   |      |
| Agona                 |          | 0.7   | 0.3      | 2.0  | 0.2      |       | 0.9     | 0.4   | 4.5     | 2.2   | 1.1     | 3.3  |          |      | 0.5    | 2.2  | 1.6   | 1.8   |       |       |       |       |      |
| Bredeney              |          |       |          |      | 0.6      |       |         |       |         |       |         |      |          |      |        |      |       |       |       | 0.4   | 0.5   |       |      |
| Heidelberg            |          | 0.6   |          |      |          |       | 0.1     | 0.3   |         | 1.3   | 1.5     |      | 1.4      |      |        |      |       | 0.7   |       | 0.9   |       | 1.1   |      |
| Derby                 |          |       | 0.4      |      |          |       | 0.5     | 0.1   |         |       |         |      |          |      |        |      |       |       |       |       |       |       |      |
| Montevideo            |          |       | 0.6      |      |          |       | 0.1     | 0.1   |         |       | 1.0     |      |          |      |        | 0.7  | 1.3   |       |       | 0.7   | 0.3   |       |      |
| Blockley              |          |       |          |      |          |       |         |       |         |       |         |      |          | 0.3  |        | 1.7  | 0.9   |       |       | 2.7   | 0.9   | 1.6   |      |
| Paratyphi B var. Java |          |       |          |      |          |       | 0.2     | 0.2   | 0.4     | 2.1   |         |      | 2.7      |      |        |      |       |       |       |       |       | 0.5   |      |
| Bovismorbificans      |          |       |          |      |          |       | 0.5     | 0.2   |         | 6.0   |         |      | 1.1      |      |        |      |       | 1.6   |       | 0.5   | 0.6   | 0.7   |      |
| Thompson              |          |       |          |      |          |       |         |       | 1.7     | 1.4   | 0.7     |      |          |      |        |      |       |       |       | 0.5   | 0.4   | 1.1   |      |
| Mbandaka              |          |       |          |      |          |       |         |       |         |       |         |      |          |      |        |      |       |       |       |       |       |       |      |
| Corvallis             |          |       |          |      |          |       |         |       |         |       |         |      |          |      |        |      |       |       |       |       |       |       |      |
| I 9,12:iv:-           |          |       |          |      |          |       |         |       |         |       |         |      |          |      |        |      |       |       |       |       |       |       |      |
| Muenchen              |          |       |          |      |          |       |         |       | 0.1     |       |         |      | 3.4      |      |        |      |       |       | 1.9   |       |       |       |      |
| Stanley               |          |       |          |      |          |       |         |       |         | 0.9   | 1.6     | 2.1  |          |      |        | 2.5  | 3.0   |       |       |       |       | 0.4   |      |
|                       |          |       |          |      |          |       |         |       |         |       |         |      |          |      |        |      |       |       |       |       |       | 2.3   |      |

\*Institutional data





Table 4. Distribution of the 20 most frequently serotyped human *Salmonella* isolates from North American countries in numbers and percentages.

| Population            | Canada     |       |       |        | United States |        |  |  |
|-----------------------|------------|-------|-------|--------|---------------|--------|--|--|
|                       | 33,212,696 |       |       |        | 303,824,640   |        |  |  |
| Year                  | 2001       | 2003  | 2007  | 2001   | 2003          | 2005   |  |  |
| Total serotyped       | 6,359      | 5,531 | 6,455 | 31,675 | 31,484        | 33,348 |  |  |
| Typhimurium           | 21.0       | 20.0  | 20.8  | 22.1   | 21.1          | 20.9   |  |  |
| Enteritidis           | 21.5       | 12.5  | 25.7  | 17.7   | 15.4          | 20.2   |  |  |
| Newport               | 2.3        | 3.3   | 2.2   | 10.0   | 12.2          | 9.9    |  |  |
| Heidelberg            | 13.9       | 19.9  | 8.7   | 5.9    | 5.7           | 5.7    |  |  |
| Javiana               |            | 1.2   |       | 3.4    | 5.3           | 4.0    |  |  |
| Montevideo            | 0.8        |       |       | 2.0    | 2.7           | 2.4    |  |  |
| Saintpaul             | 1.5        | 2.0   | 1.9   | 1.5    | 2.6           | 2.0    |  |  |
| Muenchen              |            |       | 1.3   | 1.8    | 2.5           | 2.2    |  |  |
| Thompson              | 3.7        | 2.6   | 2.7   | 1.6    | 1.6           | 1.3    |  |  |
| Oranienburg           |            | 1.7   | 2.2   | 1.9    | 1.8           | 1.8    |  |  |
| Infantis              | 1.9        | 2.2   | 2.0   | 1.4    | 1.7           | 1.5    |  |  |
| Braenderup            |            |       |       | 1.2    | 1.7           | 1.8    |  |  |
| Agona                 | 1.9        | 2.6   | 1.7   | 1.2    | 1.6           |        |  |  |
| Mississippi           |            |       |       |        | 1.4           | 1.7    |  |  |
| I 1,4,5,12:i:-        |            | 1.5   | 2.9   |        | 1.6           |        |  |  |
| Typhi                 | 1.8        | 2.4   | 2.4   | 1.1    |               |        |  |  |
| Paratyphi B var. Java | 1.0        | 1.3   |       | 1.5    |               |        |  |  |
| Hadar                 | 3.9        | 3.4   | 2.2   |        |               |        |  |  |
| Stanley               | 1.7        |       | 1.3   |        |               |        |  |  |
| Paratyphi A           |            |       | 1.5   |        |               |        |  |  |

Table 5. Distribution of the 20 most frequently serotyped human *Salmonella* isolates from Oceania countries in numbers and percentages.

|                  | Australia  |            |       | New Zealand |       |       |
|------------------|------------|------------|-------|-------------|-------|-------|
|                  | Population | 21,007,310 |       | 4,173,460   |       |       |
| Year             | 2001       | 2003       | 2001  | 2003        | 2005  | 2007  |
| Total serotyped  | 6,932      | 6,808      | 2,605 | 1,601       | 1,460 | 1,341 |
| Typhimurium      | 44.5       | 41.5       | 64.0  | 59.5        | 51.8  | 44.4  |
| Enteritidis      | 4.4        | 3.4        | 6.5   | 8.6         | 10.3  | 11.3  |
| Virchow          | 7.1        | 5.1        |       | 1.2         | 1.1   | 2.5   |
| Saintpaul        | 5.5        | 4.4        | 0.6   | 1.7         | 4.5   | 1.9   |
| Infantis         |            | 3.0        | 2.8   | 5.6         | 4.6   | 6.4   |
| Chester          | 2.6        | 3.2        |       |             |       | 2.8   |
| Birkenhead       | 3.5        | 2.6        |       |             |       |       |
| Brandenburg      |            |            | 5.3   | 3.4         | 4.7   | 3.5   |
| Bovismorbificans | 2.3        | 1.7        |       |             |       |       |
| Muenchen         | 1.9        | 2.0        |       |             |       |       |
| Hvittingfoss     | 2.2        |            |       |             |       |       |
| Heidelberg       |            |            | 4.9   | 0.7         |       |       |
| Aberdeen         | 1.9        |            |       |             |       |       |
| Anatum           |            | 1.8        |       |             |       |       |
| Typhi            |            |            | 1.0   | 1.1         | 1.9   | 3.8   |
| I 1,4,5,12:d:    |            |            | 0.5   | 1.5         |       | 1.3   |
| Mississippi      |            |            |       | 0.9         | 1.5   | 0.8   |
| Thompson         |            |            | 0.6   | 0.6         | 1.1   |       |
| Mbandaka         |            |            | 0.5   | 0.6         |       | 1.0   |
| Montevideo       |            |            |       | 2.3         |       |       |



Figure 1. Overall proportion of specific *Salmonella* serovars in 37 selected countries over time.

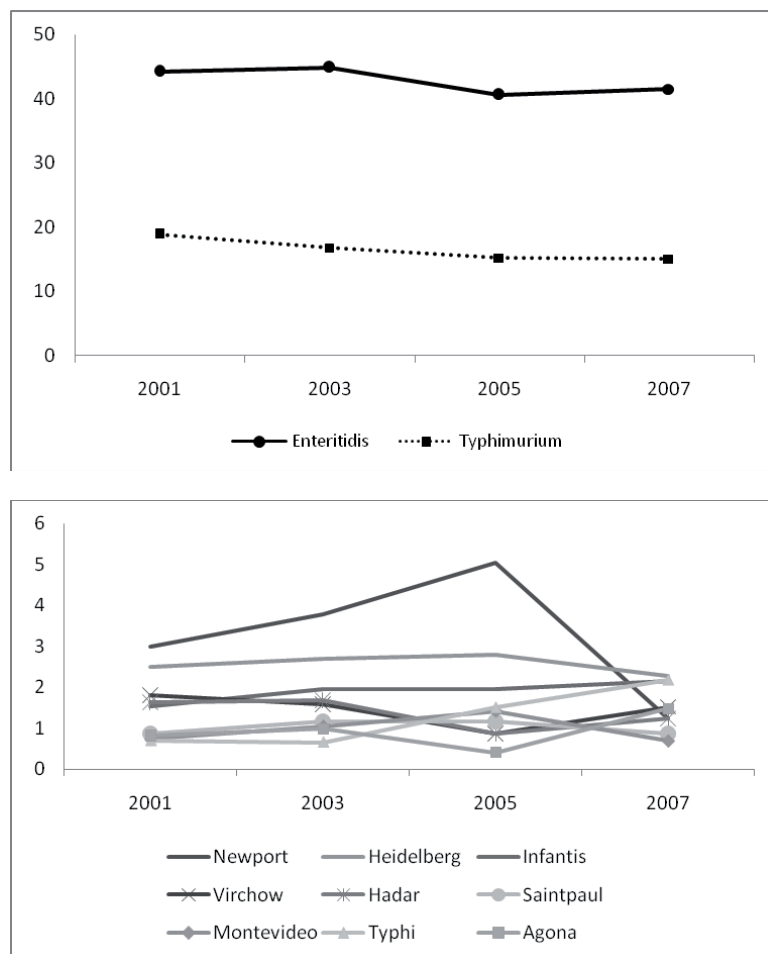


Figure 2. Proportions and trends of *S. Enteritidis* between 2001 and 2007, by region, based on data submitted by 37 selected countries.

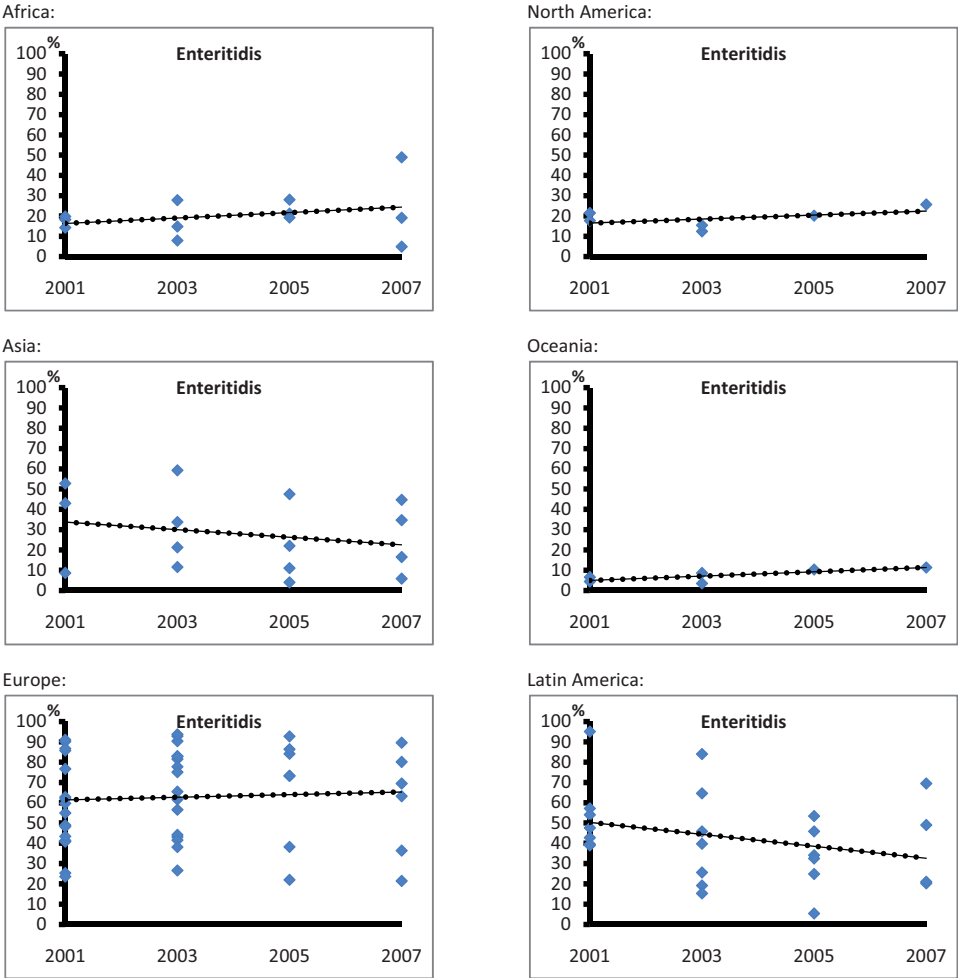


Figure 3. Proportions and trends of *S. Typhimurium* between 2001 and 2007, by region, based on data submitted by 37 selected countries.

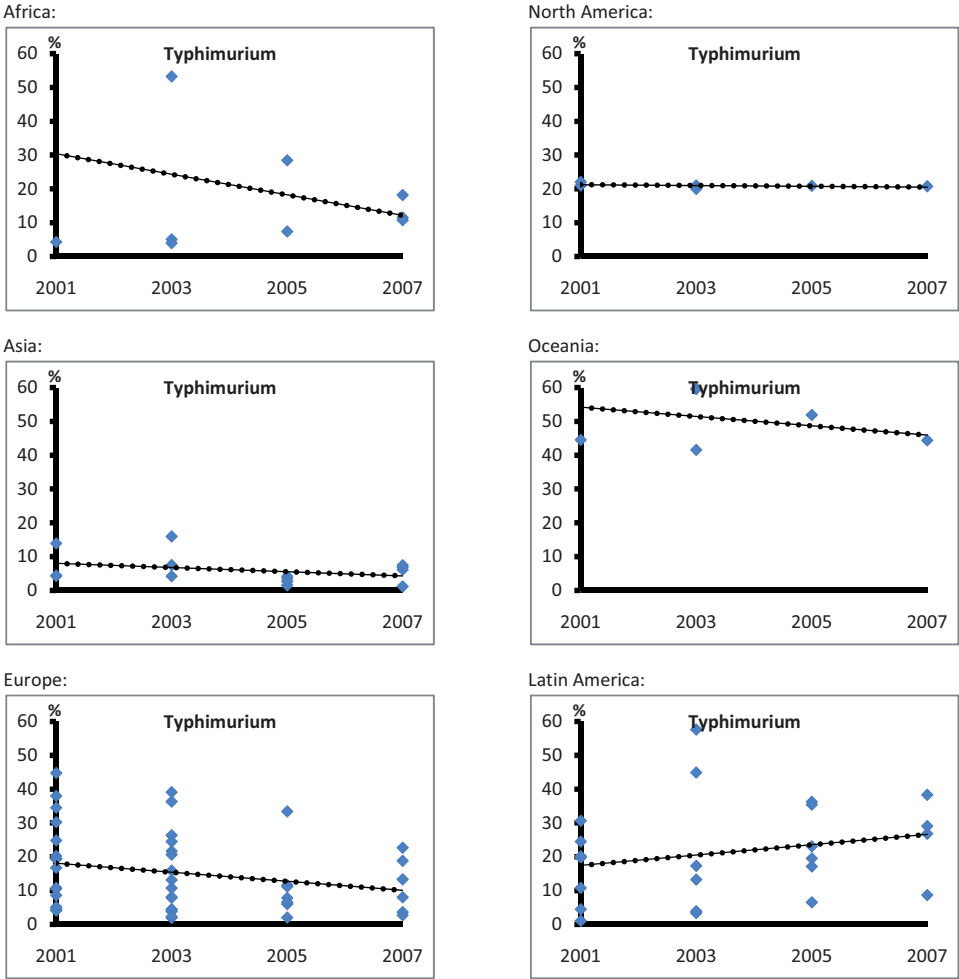
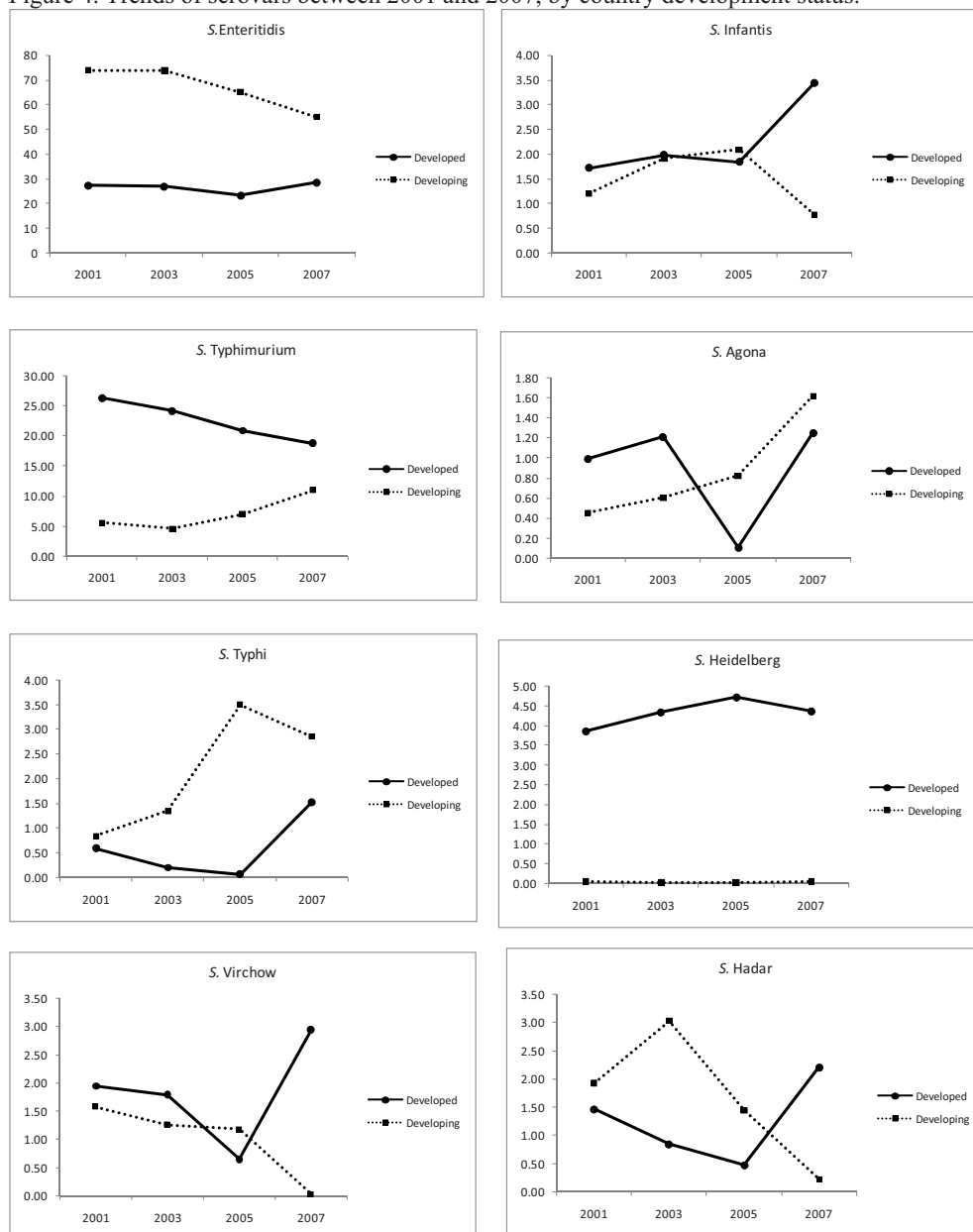


Figure 4. Trends of serovars between 2001 and 2007, by country development status.





### Article III

#### Antimicrobial resistance and molecular epidemiology of *Salmonella* Rissen from animals, food products, and patients in Thailand and Denmark.

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## Antimicrobial Resistance and Molecular Epidemiology of *Salmonella* Rissen from Animals, Food Products, and Patients in Thailand and Denmark

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### Abstract

Recently we reported increases in both the number of *Salmonella* infections due to *Salmonella* Rissen in Thailand and the isolation of this serovar from pork products in Thailand. The objectives of the present study were to determine the genetic diversity and antimicrobial resistance of *Salmonella* Rissen isolates recovered from humans, food products, and animals in Denmark and Thailand. Additionally, risk factors due to travel and consumption of specific food products were analyzed and evaluated. A total of 112 *Salmonella* Rissen isolates were included in this study from Thailand and Denmark. Thai isolates were recovered from humans, uncooked food, and ready-to-eat food. Danish isolates were obtained from humans (with and without a history of travel to Thailand prior to the infection), Danish pig or pork products, imported pig or pork products, turkeys, and animal feed. A total of 63 unique *Xba*I PFGE patterns were observed. The predominant pattern was shared by 22 strains. Limited antimicrobial resistance was observed in the Danish strains, and a higher degree of resistance was observed in strains originating from Thailand. Virtually all isolates were resistant to tetracycline. The *tetA* gene was detected in tetracycline-resistant isolates. Statistical analysis and molecular subtyping identified the combination of travel to Thailand and consumption of imported pig or pork products as well consumption of as pig or pork products produced in Denmark as risk factors for *Salmonella* Rissen infection among the Danish patients. The outcome of this study might be used as a supplement for future *Salmonella* Rissen investigations and outbreak detection.

### Introduction

*SALMONELLA ENTERICA* is a common cause of human gastroenteritis and bacteremia and a wide variety of animals, particularly food animals, have been identified as reservoirs for non-Typhi *Salmonella* (Coyle *et al.*, 1988; Humphrey *et al.*, 1988, 2000). Although 2587 serovars of *Salmonella enterica* have been identified, most human infections are caused by a limited number

of serovars. In developed countries, *Salmonella enterica* serovars Typhimurium and Enteritidis are the most common causes of human salmonellosis, although other serovars have been reported to be more prevalent in specific regions (Humphrey *et al.*, 2000; Olsen *et al.*, 2001; Herikstad *et al.*, 2002; Bangtrakulnonth *et al.*, 2004b; Galanis *et al.*, 2006). Shifts in prevalence of specific strain types and serovars can be due to international travel, human migration, and a

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global food and livestock market. Knowledge of the prevalence and molecular epidemiology of different serovars in specific regions may facilitate the recognition and control of new and emerging pathogens.

We recently reported an increase in *Salmonella* infections due to *Salmonella enterica* serovar Rissen in Thailand. The reported number of human *Salmonella* infections due to *Salmonella* Rissen in Thailand has increased from 54 cases in 1993 to 334 in 2002. During that same time period, a 10 percentage point increase (4.7% to 14.7%) in the isolation of *Salmonella* Rissen from specific food products was also observed (Bangtrakulnonth *et al.*, 2004b). While this serovar is rarely reported elsewhere in the world, it is among the top three *Salmonella* serovars found in pigs and pork products in Thailand, other Southeast Asian countries, and Spain (Bangtrakulnonth *et al.*, 2003, 2004a, 2005; Angkititrakul *et al.*, 2005; Vaeteewootacharn *et al.*, 2005; VAV, 2005; Inthavong *et al.*, 2006; Padungtod and Kaneene, 2006; Riano *et al.*, 2006; Vo *et al.*, 2006; Astorga *et al.*, 2007).

In recent years, increased antimicrobial resistance has been reported in several serovars of *S. enterica*. However, only limited data on the prevalence of antimicrobial resistance of *Salmonella* Rissen is available. In 2005, Angkititrakul reported tetracycline, streptomycin, and sulphonamide resistance among *Salmonella* Rissen isolates recovered from pork and chicken in Thailand. The isolates recovered from pork were however susceptible to ciprofloxacin, gentamicin, chloramphenicol, amoxicillin, and sulphamethoxazole + trimethoprim (Angkititrakul *et al.*, 2005). In contrast, multidrug-resistant isolates of *Salmonella* Rissen have been reported from Spain. Previous studies have shown these isolates to be resistant to ampicillin, amoxycillin, streptomycin, neomycin, sulphonamides, sulphanamides + trimethoprim, chloramphenicol, and tetracycline (Riano *et al.*, 2006; Astorga *et al.*, 2007). One study (Riano *et al.*, 2006) also described the identification of the *bla*<sub>SHV-12</sub> gene in a cefotaxime and ceftazidime resistant isolate.

Antimicrobial susceptibility profiles and molecular subtyping are useful epidemiological tools which can be used to determine the sources of an outbreak. To our knowledge, only one

molecular study of *Salmonella* Rissen has previously been undertaken. In this earlier study 19 Portuguese isolates of *Salmonella* Rissen were characterized by pulsed-field gel electrophoresis (PFGE). Three unique *Xba*I patterns were observed, with the predominant pattern being shared by 17 isolates (Vieira-Pinto *et al.*, 2006).

The objectives of the present study were to determine the genetic diversity and antimicrobial resistance of *Salmonella* Rissen isolates recovered from humans, food products, and animals in Denmark and Thailand.

Microbiological findings were correlated with epidemiological data, and risk factors for infection with *Salmonella* Rissen were calculated.

The outcome of this study might be used as a supplement for future *Salmonella* Rissen investigations and outbreak detection.

## Methods

### Bacterial isolates

The WHO National *Salmonella* and *Shigella* Centre in Bangkok receives all presumptive positive *Salmonella* isolates from all diagnostic laboratories throughout Thailand. In 2004, 300 isolates were identified as *Salmonella* Rissen. The isolates originated from 9 of 12 districts and Bangkok.

In addition to human isolates, The WHO National *Salmonella* and *Shigella* Centre also receives isolates recovered from food products. In 2004, the centre received 295 isolates, originating from various food sources. The majority of the isolates originated from pork products. These samples were further characterized as "raw food" ( $n = 128$ ) or "ready-to-eat food" ( $n = 92$ ). The raw food isolates were submitted from districts 1, 3, and 4 and Bangkok over a 1-year period, excluding the months of February to April and October to December. The isolates originating from ready-to-eat food were collected over a 9-month period (April–December) from districts 1 and 10 and Bangkok.

The 23 human isolates from Denmark were collected between 2000 and 2005 by the National Reference Laboratory for Enteropathogenic Bacteria, Statens Serum Institut (SSI) in Denmark. These isolates were recovered from patients suffering from gastrointestinal infections caused by *Salmonella* Rissen. Four cases were

acquired in Denmark. Six patients had traveled to Thailand prior to becoming ill. Travel history was unavailable for the remaining 13 cases. An additional 155 isolates were identified by the National Food Institute, Copenhagen, Denmark. These isolates were obtained from pig or pork products of Danish origin ( $n = 61$ ), imported pig or pork products ( $n = 19$ ), turkey ( $n = 19$ ), and animal feed ( $n = 24$ ). The remaining 32 isolates were either from an unknown source or originated from caged birds, chickens, the environment, horses, cattle, or bone meal. All isolates were collected between 1996 and 2005.

The isolates were initially identified as *Salmonella* spp. according to internationally recognized procedures. Isolates were then serotyped using slide agglutination in the country of origin.

#### Selection of isolates

Using a stratified random sampling technique, 33 of the 300 human isolates from Thailand were selected for additional characterization. Isolates were from both male and female patients residing in districts 1, 4, 5, 7, 8, and 12 and Bangkok. Patients ranged in age from 1 to 42 years. A review of the sample collection date was used to insure that the isolates were representative of the study period. The isolates from the raw food and ready-to-eat food were selected from the same time period as the Thai human isolates. The majority of the food isolates were recovered in May and June.

Thirteen of the 128 raw food isolates were selected for additional characterization. The selected isolates were from districts 1, 3, and 4 and Bangkok. Ten of the ninety-two isolates from ready-to-eat food were selected for additional characterization. The selected isolates were from district 1 and Bangkok.

All 23 human isolates from Denmark were included in the study. A stratified random sampling technique was used to select isolates from the NFI collection for additional characterization.

Isolates selected for additional characterization were obtained from: Danish pigs and pork products collected between 2002 to 2005 ( $n = 10$ ); imported pig or pork products (Spanish origin  $n = 5$ , German origin  $n = 1$ , unknown origin  $n = 3$ ) submitted to Danish import control au-

thorities between 2002 and 2005 ( $n = 9$ ); turkey collected between 2000 and 2002 ( $n = 6$ ); and animal feed collected between 2001 and 2005 ( $n = 8$ ).

#### Risk factors

SAS version 9.1.3 (SAS Institute Inc., Cary, NC) was used to calculate the relative risk using a Fisher's Exact test. The risk of disease for consumption of imported pork from Spain and the risk factor of travel to Thailand among the Danish patients infected with *Salmonella* Rissen were evaluated.

#### Serotyping

O and H antigens were characterized by agglutination with hyperimmune sera and serotype was assigned according to the Kauffmann-White scheme (Popoff and Le Minor, 2001).

#### Antimicrobial susceptibility testing

Susceptibility to antimicrobial agents was performed at the NFI on all selected isolates as minimum inhibitory concentration determinations using a commercially prepared, dehydrated panel (Sensititre<sup>®</sup>) from TREK Diagnostic Systems Ltd. (East Grinstead, England).

The following antimicrobials and resistance cut-off values or breakpoint were used in the study: ampicillin, AMP ( $R > 4 \mu\text{g/mL}$ ); amoxicillin + clavulanic acid, AUG ( $R > 4 \mu\text{g/mL}$ ); apramycin, APR ( $R > 16 \mu\text{g/mL}$ ); cefalothin, CEF ( $R > 16 \mu\text{g/mL}$ ); cefpodoxime, POD ( $R > 1 \mu\text{g/mL}$ ); ceftiofur, XNL ( $R > 2 \mu\text{g/mL}$ ); chloramphenicol, CHL ( $R > 16 \mu\text{g/mL}$ ); ciprofloxacin, CIP ( $R > 0.06 \mu\text{g/mL}$ ); colistin COL ( $R > 8 \mu\text{g/mL}$ ); florfenicol, FFN ( $R > 2 \mu\text{g/mL}$ ); gentamicin, GEN ( $R > 2 \mu\text{g/mL}$ ); nalidixic acid, NAL ( $R > 16 \mu\text{g/mL}$ ); neomycin, NEO ( $R > 8 \mu\text{g/mL}$ ); spectinomycin, SPE ( $R > 64 \mu\text{g/mL}$ ); streptomycin, STR ( $R > 16 \mu\text{g/mL}$ ); sulphamethoxazole, SMX ( $R > 256 \mu\text{g/mL}$ ); tetracycline, TET ( $R > 8 \mu\text{g/mL}$ ); and trimethoprim, TMP ( $R > 2 \mu\text{g/mL}$ ).

Epidemiological cut-off values were interpreted according to current eucast (<http://www.eucast.org>) and European Food Safety Authority (EFSA) recommendations. Exceptions were made for interpretation of APR, AUG, CEF,

COL, FFN, POD, SPE, and XNL, where Clinical and Laboratory Standards Institute (CLSI, 2002, 2006a, 2006b) guidelines and clinical breakpoints were utilized. NEO and STR were interpreted according to research conducted at NFI. Quality control using *E. coli* ATCC 25922 was conducted on a weekly basis according to CLSI.

#### Detection of resistance genes

Resistant and intermediate strains were further characterized through the use of a polymerase chain reaction (PCR) assay with primers specific for 25 antimicrobial resistance genes (Table 1).

All PCR amplifications were performed with buffer supplied by the manufacturer, 20 pmol/ $\mu$ L of each primer and 0.5 U of Amplicon Taq Polymerase (Ampliqon, Copenhagen, Denmark). The final reaction volume was (50  $\mu$ L). The following cycling conditions were used for all reactions: 3 minutes at 94°C; 35 cycles of 1 minute at 94°C, 1 minute at the appropriate annealing temperature, and 1 minute at 72°C; 10 minutes at 72°C. Primer names and sequences, resistance genes, primer position, annealing temperature, control strains, amplicon sizes, and references are listed in Table 1.

Amplicons produced by the seven CIP-resistant strains and the single *bla*<sub>CTX</sub> amplicon generated, were selected for sequencing. Prior to sequencing, the amplicons were purified using the GFX<sup>TM</sup> PCR DNA kit (Amersham Biosciences, Piscataway, NJ). The DNA was shipped to MacroGen Inc. (Seoul, Korea) for sequencing using the same primers as in the PCR analysis. Sequence analysis and alignment was performed using Vectors NTI suite 9 (InforMax, Inc., Bethesda, MD) software. The resulting nucleotide sequences were compared to sequences obtained from the GenBank database (<http://www.lahey.org/studies/webt.html>).

#### Pulsed-field gel electrophoresis

The selected isolates were analyzed for genetic relatedness by PFGE using *Xba*I according to the Centers for Disease Control and Prevention (CDC) PulseNet protocol (Ribot *et al.*, 2002). The electrophoresis was performed with a CHEF DR III System (Bio-Rad Laboratories, Hercules, CA) by using 1% SeaKem agarose in 0.5 $\times$ Tris-

borate-EDTA at 180 V. Running conditions consisted of 1 phase from 2.2 to 63.8 seconds for a run time of 22 hours.

PFGE following digestion with *Bln*I was utilized to further differentiate the 22 isolates which exhibited the predominant *Xba*I pattern (TEEX01.0017.DK). Comparison of the PFGE profiles was performed by using Bionumerics software version 4.6 (Applied Maths, Sint-Martens-Latem, Belgium) and the dice correlation for band matching with a 1.0% position tolerance and an optimization at 1.0% using both *Xba*I and *Bln*I.

## Results

#### Risk factors

Available travel histories suggested that six Danish patients (5%) may have acquired their infection abroad and four patients (4%) may have obtained the infection in Denmark. All six patients (5%) who traveled to Thailand shared a common PFGE pattern with a Thai isolate. Six (5%) of the remaining 17 patients (15%) did not share a common PFGE pattern. Among the Danish patients, there did not appear to be a statistically significant connection using Fisher's Exact test ( $p = 0.1438$ ) between travel to Thailand (exposure) and sharing a common PFGE profile with a Thai source (outcome).

Nine isolates (8%) included in the study originated from imported pig or pork products. Six of these isolates (5%) shared a common PFGE pattern with a Danish patient. Three (3%) of the ten Danish pig or pork product samples (9%) included in this study shared a common PFGE pattern with a Danish patient. The risk of consuming imported pig or pork (exposure) was estimated between pork or pig and sharing a common PFGE profile with Danish patients who had not traveled prior to the onset of infection (outcome). No statistical significance was observed using Fisher's Exact test ( $p = 0.3469$ ).

#### Antimicrobial resistance

All eight (7%) isolates from animal feed originating from Denmark were fully susceptible to all antimicrobials tested. A similarly low frequency of resistance was observed in six (5%)

TABLE 1. OLIGONUCLEOTIDE PRIMER SEQUENCES USED FOR THE AMPLIFICATION OF THE VARIOUS RESISTANCE GENES

| Antimicrobial family          | Resistant gene      | Primer name   | Primer no. | Sequence                                     | Position  | Annealing temp. (°C) | Control strain                          | Amplicon size (bp) | Reference                      |
|-------------------------------|---------------------|---|------------|--|-----------|----------------------|---|--------------------|--------------------------------|
| Beta-lactams                  | <i>bla</i> CTX      | ctx-M-9 P1  | 1096       | 5'-GTC ACA AAG AGA GTG CAA CGC-3'            | 85-105    | 60                   | <i>Salmonella</i> Vinchow               | 857                | Costa et al., 2006             |
|                               |                     | ctx-M-9 P2  | 1097       | 5'-ATG ATT CTC GCC GCT GAA GGC-3'            | 941-921   |                      | 75-22438-1                              |                    |                                |
|                               |                     | ctx M U1  | 1354       | 5'-ATG TCG AGY ACC AGT AAR GTG ATG GC-3'     | 211-236   |                      | <i>Salmonella</i> Vinchow               |                    |                                |
| Beta-lactams                  | <i>bla</i> CTX      | ctx-M-U2  | 1355       | 5'-TGG GTR AAR TAR GTS ACC AGA ATC AGC GC-3' | 803-775   | 60                   | 75-22438-1                              | 593                | 2002; Archambault et al., 2006 |
| Beta-lactams                  | <i>bla</i> TEM      | TEM-C-Rev   | 686        | 5'-ATG ATT GCT TAA TCA GTG AG-3'             | 2671-2652 | 50                   | <i>Salmonella</i> Paratyphi B           | 586                | Pateron et al., 2003           |
|                               |                     | Pr622 tem 1A primer 2                               | 690        | 5'-ACC ATA CCG AGG GCT GTC GC-3'             | 2086-2107 |                      | TEM20                                   |                    |                                |
| Aminoglycosides               | <i>aac</i> (3'-HV)  | AME 14ACC(3) IV F                                   | 152        | 5'-AGT TGA CCC AGG GCT GTC GC-3'             | 938-919   | 63                   | <i>E. coli</i>                          | 627                | Maynard et al., 2003           |
|                               |                     | AME 14ACC(3) IV F                                   | 153        | 5'-GTG TCG TCG TCG TCC ACA GC-3'             | 311-330   |                      | <i>E. coli</i>                          |                    |                                |
| Aminoglycosides               | <i>aac</i> (2'-I)   | AME 3, ANT (2'-I)-1                                 | 81         | 5'-GGG CGG GTC ATC GAG GAG TT-3'             | 1491-1510 | 67                   | <i>E. coli</i>                          | 329                | Cameron et al., 1986           |
|                               |                     | AME 4, ANT (2'-I)-1                                 | 82         | 5'-TAT CGC CTG AAA GCG GC-3'                 | 1819-1800 |                      | K2 ANT (2'-I)                           |                    |                                |
| Aminoglycosides               | <i>aac</i> (3'-H)   | AME7 AAC(3)-H1 X51534 381->400                      | 87         | 5'-TGA AAC GCT GAA GCG TC-3'                 | 471-490   | 55                   | <i>Klebsiella</i>                       | 370                | Vliegberth et al., 1989        |
|                               |                     | AME8 AAC(3)-H1 X51534 759->731                      | 88         | 5'-TGA AAC GCT GAA GCG TC-3'                 | 840-821   |                      | K8 AAC(3)-H1                            |                    |                                |
| Aminoglycosides               | <i>aph</i> (3'-III) | P1-APH(3'-III) new                                  | 203        | 5'-GGC GAT GTG GAT GCG AA-3'                 | 454-473   | 52                   | <i>Enterococci</i>                      | 292                | Lido et al., 2006              |
|                               |                     | P2-APH(3'-III)                                      | 1428       | 5'-GCT GTC TCG CTA GTC GTC GCG-3'            | 745-726   |                      | JH2-1.5 APH (3'-III) obtained amplicons |                    |                                |
| Aminoglycosides               | <i>aph</i> (3'-I)   | AME 19 APH(3') I-F                                  | 171        | 5'-AAC GTC TCG CTA GTC GTC TCG GC-3'         | 1182-1202 | 68                   | optained amplicons                      | 670                | Oka et al., 1981               |
|                               |                     | AME 20 APH(3') I-F                                  | 172        | 5'-GCT ATT CGG CTA TGA CTG GGC-3'            | 768-788   |                      | optained amplicons                      |                    |                                |
| Aminoglycosides               | <i>aph</i> (3'-H)   | AME 22 APH (3')-I-F                                 | 186        | 5'-CCA CCA TAT TCG CCA ACC-3'                | 1309-1289 | 60                   | <i>Salmonella</i> Typhimurium           | 542                | Aarestrup et al., 2003         |
|                               |                     | AME 23 APH (3')-I-F                                 | 187        | 5'-CCT CTG ATG AAT GCT CAT CCG-3'            | 569-580   |                      | DS611                                   |                    |                                |
| Phenicolis                    | <i>catA</i> 1       | CAT2X6410a 193-213                                  | 94         | 5'-CCT CTG ATG AAT GCT CAT CCG-3'            | 126-145   | 60                   | <i>Salmonella</i> Typhimurium           | 455                | Aarestrup et al., 2003         |
|                               |                     | CAT2X6410a 649-630                                  | 95         | 5'-TAC TCG AAG ACC GTC TGG CC-3'             | 2028-2047 |                      | DS611                                   |                    |                                |
| Phenicolis                    | <i>catA</i> A       | CmlA 1F, U12338 3860=>3879                          | 114        | 5'-TTC TCG AAG ACC GTC ATT GC-3'             | 2586-2605 | 65                   | <i>Salmonella</i> Typhimurium           | 578                | In this study                  |
|                               |                     | CmlA 2B, U12338 4437=>4418                          | 115        | 5'-CAG CTT GGT CTT CAA CTG CA-3'             | 353-372   |                      | DS611                                   |                    |                                |
| Phenicolis                    | <i>fla</i> R        | PO-FLOK NY  | 398        | 5'-ACG GAC ACC GTG AAG ACA AT-3'             | 1024-1043 | 60                   | <i>Salmonella</i> Typhimurium           | 691                | In this study                  |
|                               |                     | Flac-3-sev  | 437        | 5'-TAC CGT GAT AGCTAT CCA CGA-3'             | 305-325   |                      | DS611                                   |                    |                                |
| Quinolones                    | <i>gyr</i> A        | gyrA_P2 bio   | 184        | 5'-GTA CTT TAC CGG ATG AAC GT-3'             | 13-32     | 60                   | <i>Salmonella</i>                       | 313                | Wuuf et al., 2000              |
|                               |                     | gyrA_P3   | 185        | 5'-CCA ATC GCA GAT AGA AGC C-3'              | 787-769   |                      | PS02212                                 |                    |                                |
| Aminoglycosides               | <i>str</i> A        | Strept-res, strA, plasmi-ref1010/787-769 primer-2   | 67         | 5'-CTT GGT GAT AAC GGC AAT TC-3'             | 240-259   | 55                   | <i>E. coli</i>                          | 548                | Aarestrup et al., 2003         |
|                               |                     | Strept-res, strA, plasmi-ref1010/240-259 primer-1   | 68         | 5'-GGA TCG TAG AAC ATA TTG GC-3'             | 623-604   |                      | RF1010                                  |                    |                                |
| Aminoglycosides               | <i>str</i> B        | Strept-res, strB, plasmi-ref1010, 623-604, primer-2 | 65         | 5'-GGA TCG TAG AAC ATA TTG GC-3'             | 623-604   | 56                   | <i>E. coli</i>                          | 509                | Scholz et al., 1989            |
|                               |                     | Strept-res, strB, plasmi-ref1010, 115-134, primer-1 | 66         | 5'-ATC GTC AAG GGA TTG AAA CC-3'             | 115-134   |                      | RF1010                                  |                    |                                |
| Aminoglycosides               | <i>aad</i> A        | aadA forward  | 521        | 5'-ATT TCG TGG TTA CGG TGA CC-3'             | 234-253   | 56                   | <i>Salmonella</i>                       | 534                | In this study                  |
|                               |                     | aadA backward                                       | 522        | 5'-CTT CAA GTC TGA CGG GCT GA-3'             | 748-767   |                      | PS02212                                 |                    |                                |
| Quaternary ammonium compounds | <i>qacEΔ1</i>       | des-1   | 847        | 5'-ATC GCA ATA GTT GGC GAA GT-3'             | 2529-2548 | 57                   | <i>Salmonella</i> Typhimurium           | 226                | Sandvang et al., 1997          |
|                               |                     | des-2   | 848        | 5'-CAA GCCTTT GCC CAT GAA GC-3'              | 2735-2754 |                      | DS611                                   |                    |                                |
| Sulfonamides                  | <i>su</i> I         | Sul 1 forward                                       | 519        | 5'-TGA GAT CAG AGT TAT TCC GC-3'             | 2875-2894 | 58                   | <i>Salmonella</i>                       | 406                | In this study                  |
|                               |                     | Sul 1 backward                                      | 520        | 5'-TTG AAG GTT CGA CAC GT-3'                 | 3261-3280 |                      | PS02212                                 |                    |                                |
| Sulfonamides                  | <i>su</i> II        | Sul 2-f   | 591        | 5'-GGC CTC AAG GCA GAT GGC ATT-3'            | 154-174   | 68                   | <i>Salmonella</i> Typhimurium           | 285                | Aarestrup et al., 2003         |
|                               |                     | Sul 2-b   | 592        | 5'-GGC TTT GAT ACC GGC ACC CCG-3'            | 418-438   |                      | B(Sul2)                                 |                    |                                |
| Sulfonamides                  | <i>su</i> III       | Sul3(f)   | 1356       | 5'-GAG CAA GAT TTT TGG AAT CC-3'             | 2981-3000 | 51                   | <i>Salmonella</i> Typhimurium           | 773                | Perraton and Boerlin, 2003     |
|                               |                     | Sul3(B)   | 1357       | 5'-CAT CTC CAG CTA ACC TAG GC-3'             | 3770-3753 |                      | C(Sul3)                                 |                    |                                |
| Tetracycline                  | <i>tet</i> A        | TetA primer1  | 173        | 5'-GTA ATT CTG ACC ACT GTC GC-3'             | 1084-1103 | 57                   | <i>E. coli</i>                          | 957                | Aarestrup et al., 2003         |
|                               |                     | TetA primer2  | 175        | 5'-CTC CCT GGA CAA CAT TCG TT-3'             | 2021-2040 |                      | NCTC 50078                              |                    |                                |
| Tetracycline                  | <i>tet</i> B        | TetB-Tet10=-39->58                                  | 90         | 5'-CTC AGT CCA CCA AGC TTG GC-3'             | 39-58     | 52                   | <i>E. coli</i>                          | 415                | Aarestrup et al., 2003         |
|                               |                     | TetB-Tet10=-454->434                                | 91         | 5'-CTC AGT CCA CCA AGC TTG GC-3'             | 454-434   |                      | CSH9-Th10                               |                    |                                |
| Tetracycline                  | <i>tet</i> C        | TetC-595->576                                       | 92         | 5'-GCT TCA AGC CTC TCA AGG GC-3'             | 595-576   | 62                   | <i>E. coli</i>                          | 506                | Sengulov et al., 2003          |
|                               |                     | TetC-90->110  | 93         | 5'-GCT TCA AGC CTC TCA AGG GC-3'             | 90-110    |                      | D07-pBR322                              |                    |                                |
| Tetracycline                  | <i>tet</i> D        | Tet D2 (894-874)                                    | 575        | 5'-GTA TCG GGA AGT GAT AGC-3'                | 894-874   | 57                   | <i>E. coli</i>                          | 436                | Aarestrup et al., 2003         |
|                               |                     | Tet D1 (469-478)                                    | 576        | 5'-GGA TAT CTC ACC GCA TCT GC-3'             | 439-478   |                      | C600-psL106                             |                    |                                |
| Tetracycline                  | <i>tet</i> G        | Tet(G)-1  | 229        | 5'-GCA GCG AAA CGG TAT TTG CC-3'             | 29-48     | 60                   | <i>Salmonella</i>                       | 663                | Aarestrup et al., 2003         |
|                               |                     | Tet(G)-2  | 230        | 5'-TCC GAA AGC TGT CCA ACC AT-3'             | 672-691   |                      | PS02212                                 |                    |                                |

TABLE 2. ORIGIN AND FREQUENCY OF RESISTANCE AMONG *SALMONELLA* RISEN ISOLATES FROM VARIOUS SOURCES IN DENMARK AND THAILAND

| Country/Source | No. (%) of isolates      | No. (%) of resistant isolates against the listed antimicrobials |     |         |        |        |       |        |        |        |        |        |        |         |        |         |         |        |
|----------------|--------------------------|---|-----|---------|--------|--------|-------|--------|--------|--------|--------|--------|--------|---------|--------|---------|---------|--------|
|                |                          | AUG   | AMP | APR     | CEF    | POD    | XNL   | CHL    | CIP    | FFN    | GEN    | NAL    | NEO    | SPE     | STR    | SMX     | TET     | TMPP   |
| Denmark        | Patients <sup>a,b</sup>  | 17 (15)   | 0   | 2 (12)  | 0      | 1 (6)  | 1 (6) | 1 (6)  | 1 (6)  | 0      | 0      | 1 (6)  | 0      | 2 (12)  | 2 (12) | 2 (12)  | 14 (82) | 2 (12) |
|                | Turisme (Thailand)       | 6 (5)   | 0   | 3 (50)  | 0      | 0      | 0     | 2 (33) | 1 (17) | 0      | 0      | 1 (17) | 1 (17) | 1 (17)  | 3 (50) | 1 (17)  | 6 (100) | 1 (17) |
|                | Turkey <sup>c</sup>      | 6 (5)   | 0   | 0       | 0      | 0      | 0     | 0      | 0      | 0      | 0      | 0      | 0      | 0       | 1 (17) | 0       | 1 (17)  | 0      |
|                | Animal feed              | 8 (7)   | 0   | 0       | 0      | 0      | 0     | 0      | 0      | 0      | 0      | 0      | 0      | 0       | 0      | 0       | 0       | 0      |
|                | Pig/Pork                 | 10 (9)  | 0   | 2 (20)  | 0      | 0      | 0     | 0      | 0      | 0      | 0      | 0      | 1 (10) | 2 (20)  | 2 (20) | 2 (20)  | 8 (80)  | 2 (20) |
| Thailand       | Imp. Pork                | 9 (8)   | 0   | 2 (22)  | 1 (11) | 0      | 0     | 0      | 0      | 0      | 1 (11) | 0      | 0      | 2 (22)  | 3 (33) | 3 (33)  | 9 (100) | 1 (11) |
|                | Patients                 | 33 (30)   | 0   | 12 (36) | 0      | 1 (3)  | 0     | 9 (27) | 5 (15) | 1 (3)  | 2 (6)  | 5 (15) | 4 (12) | 11 (33) | 9 (27) | 10 (30) | 29 (88) | 9 (27) |
|                | Ready-to-eat food (Pork) | 10 (9)  | 0   | 3 (30)  | 0      | 1 (10) | 0     | 2 (20) | 0      | 1 (10) | 0      | 0      | 2 (20) | 3 (30)  | 1 (10) | 3 (30)  | 8 (80)  | 3 (30) |
|                | Raw food (Pork)          | 13 (12)   | 0   | 5 (38)  | 0      | 0      | 0     | 2 (15) | 0      | 0      | 0      | 0      | 0      | 5 (38)  | 2 (15) | 5 (38)  | 10 (77) | 5 (38) |

<sup>a</sup>Including one isolate from a premature infant that was resistant to AMP, CEF, POD, XNL, CHL, SPE, STR, SMX, TET, and TMP.

<sup>b</sup>Travel histories were available for 9/23 Danish patients.

<sup>c</sup>Samples include: meat, animal feed and live animals.

AMP<sup>a</sup>, ampicillin; AUG, amoxicillin + clavulanic acid; APR, apramycin; CEF, cefalothin; POD, cepodoxime; XNL, ceftiofur; CHL, chloramphenicol; CIP, ciprofloxacin; FFN, florfenicol; GEN, gentamicin; NAL, nalidixic acid; NEO, neomycin; SPE, spectinomycin; STR, streptomycin; SMX, sulphamethoxazole; TET, tetracycline; TMP, trimethoprim.

isolates from turkey slaughtered in Denmark and 10 (9%) isolates from pig or pork products produced in Denmark (Table 2).

Danish-produced pig or pork product isolates and isolates from imported pig or pork products had a higher frequency of resistance to TET (80.0 %).

A higher frequency of resistance was observed in the six isolates (5%) cultured from Danish patients with a history of travel to Thailand compared to the 17 isolates (15%) from Danish patients. Resistance to CHL, CIP, NAL, NEO, SPE, SMX, and TMP ranged from 17% to 33%. A higher frequency of resistance was seen to AMP, STR, and TET with 50%, 50%, and 100% resistance observed, respectively.

The human isolates from Thailand showed high resistance to AMP (36%), CHL (27%), SPE (33%), STR (27%), SMX (30%), and TMP (27%). The majority of isolates (88%) were fully resistant to TET.

The isolates from raw food and ready-to-eat food showed similar trends in the frequency of resistance. High level of resistance was seen against TET (77–80%) (Table 2). One human isolate from Denmark found to be multidrug resistant: AMP, CEP, POD, XNL, CHL, SPE, STR, SMX, TET, and TMP. However, this strain was recovered from a premature infant who most likely had received treatment with antimicrobials.

#### Identification of resistance genes

*TetA* specific amplicons were produced by all TET-resistant isolates (18%) obtained from Danish patients; including those with a history of travel to Thailand (8%), pork imported from Spain or Germany (7%), ready-to-eat food (9%), and raw food from Thailand; and five isolates from Danish pig or pork products (4%). The three isolates (3%) from Danish pig or pork products, and one isolate (1%) from turkey also yielded *tetB* specific amplicons (Table 3).

All SMX-resistant isolates (2%) obtained from Danish patients produced amplicons specific to *sul1*. The SMX-resistant isolate (1%) from Danish patient with a history of travel to Thailand produced a *sul3* specific amplicon. Two isolates (2%) from Danish pig or pork product samples produced *sul1* and *sul2* specific amplicons, respec-

tively. The three isolates (3%) from imported pig or pork products and the ready-to-eat (3%) and raw food (4%) samples from Thailand produced amplicons to *sul1* and *sul3* (Table 3).

A single base-pair substitution in the *gyrA* gene was identified in seven CIP-resistant strains (7%). Five of these strains (4%) originated from Thai patients and two (2%) from Danish patients, one (1%) of whom had travelled to Thailand. Isolates from three of the Thai patients (3%) and one of the Danish patients (1%) had a mutation at codon 83 (TCC [Ser] → TTC [Phe]). The two remaining Thai patients (2%) and the Danish patient (1%) (who had travelled to Thailand) had a mutation at codon 87 (GAC [Asp] → AAC [Asn]). (Mutated bases are shown in bold.)

The isolate from the Danish patient which produced a positive amplicon to *bla*<sub>CTX</sub> was sequenced and showed 100% similarity to strains in the GenBank encoding for *bla*<sub>CTX-M-14</sub>.

#### PFGE typing

A total of 63 unique *XbaI* patterns; were observed among the 112 isolates (Fig. 1). These 63 patterns formed 10 distinct clusters. All isolates from Danish patients with a history of travel to Thailand (*n* = 6) shared *XbaI* patterns with isolates recovered in Thailand. Five of these isolates matched patterns obtained from isolates recovered from raw food or ready-to-eat food samples in Thailand. The predominant pattern TEEX01.0017.DK belonged to PFGE cluster 4 and consisted of 22 (20%) isolates. This *XbaI* cluster included: five isolates (4%) from Thai patients; three isolates (3%) from ready-to-eat food; two isolates (2%) from raw food; six isolates (5%) from Danish patient, two (2%) of whom had traveled to Thailand; four isolates (4%) from imported pig or pork products (*n* = 3 from Spain and *n* = 1 from Germany), and two isolates (2%) originating from Danish pig or pork products. When pattern TEEX01.0017.DK was further subtyped using the *BlnI* enzyme, six patterns (TEEA26.0001.DK–TEEA26.0006.DK) were obtained (Fig. 2). The predominant pattern TEEA26.0001.DK was shared by 17 isolates (15%) including: six isolates (5%) from Danish patients, two (2%) of whom had traveled to Thailand; one pig or pork product isolate (1%)



TABLE 3. DISTRIBUTION OF ANTIMICROBIAL RESISTANCE GENES IN *SALMONELLA* RISSEN ISOLATES FROM DIFFERENT SOURCES

| No. (%) of isolates for each gene |              |           |          |          |           |                 |         |              |          |            |            |             |              |           |                              |                  |            |            |                                     |           |           |           |            |            |       |
|-----------------------------------|--------------|-----------|----------|----------|-----------|-----------------|---------|--------------|----------|------------|------------|-------------|--------------|-----------|------------------------------|------------------|------------|------------|-------------------------------------|-----------|-----------|-----------|------------|------------|-------|
| Country/Source                    | Tetracycline |           |          |          |           | Chloramphenicol |         | Florphenicol | Neomycin | Gentamicin | Quinolones |             | Streptomycin |           | Spectinomycin & Streptomycin | Sulfamethoxazole |            |            | Quaternary ammonium compounds (QAC) |           |           |           |            |            |       |
|                                   | tet A        | tet B     | tet C    | tet D    | tet G     | cmd A           | cat A1  |              |          |            | flo R      | aph(3')-III | aph(3')-II   | aph(3')-I |                              | aac(3')-IV       | ent(2')-II | aac(3')-II | gyr A                               | str A     | str B     | aad A     | sul 1      | sul 2      | sul 3 |
| Denmark                           | 14/14 (100)  | 0/14 (0)  | 0/14 (0) | 0/14 (0) | 0/2 (0)   | —               | —       | —            | —        | —          | —          | —           | —            | —         | 17/1 (100)                   | 0/3 (0)          | 0/3 (0)    | 2/3 (67)   | 2/2 (100)                           | 0/2 (0)   | 0/2 (0)   | 2/2 (100) | 1/2 (50)   | 14/1 (100) | —     |
| Patients <sup>a</sup>             | 6/6 (100)    | 0/6 (0)   | 0/6 (0)  | 0/6 (0)  | 1/1 (100) | 1/1 (100)       | 0/1 (0) | 0/1 (0)      | —        | —          | —          | —           | —            | —         | 17/1 (100)                   | 2/4 (50)         | 2/4 (50)   | 1/4 (25)   | 0/1 (0)                             | 0/1 (0)   | 1/1 (100) | 0/1 (0)   | 3/3 (100)  | —          |       |
| Turkine                           | 0/1 (0)      | 1/1 (100) | 0/1 (0)  | 0/1 (0)  | —         | —               | —       | —            | —        | —          | —          | —           | —            | —         | —                            | 1/2 (50)         | 1/2 (50)   | 0/2 (0)    | —                                   | —         | —         | —         | —          | —          |       |
| (Thailand)                        | 5/8 (63)     | 3/8 (37)  | 0/8 (0)  | 0/8 (0)  | 0/8 (0)   | —               | —       | —            | —        | —          | —          | —           | —            | —         | —                            | 2/5 (40)         | 1/5 (20)   | 2/5 (40)   | 1/2 (50)                            | 1/2 (50)  | 0/2 (0)   | 2/2 (100) | 2/2 (100)  | —          |       |
| Animal feed                       | 9/9 (100)    | 0/9 (0)   | 0/9 (0)  | 0/9 (0)  | 0/9 (0)   | —               | —       | —            | —        | —          | —          | —           | —            | —         | —                            | 2/4 (50)         | 2/4 (50)   | 2/4 (50)   | 1/3 (33)                            | 0/3 (0)   | 2/3 (66)  | 1/3 (33)  | 2/2 (100)  | —          |       |
| Human feed                        | 29/29 (100)  | 0/29 (0)  | 0/29 (0) | 0/29 (0) | 5/11 (45) | 11/11 (100)     | 0/3 (0) | 0/3 (0)      | 0/3 (0)  | 4/5 (80)   | 0/5 (0)    | 0/2 (0)     | 0/2 (0)      | 2/2 (100) | 5/5 (100)                    | 4/14 (29)        | 4/14 (29)  | 11/15 (73) | 6/10 (60)                           | 1/10 (10) | 5/10 (50) | 6/10 (60) | 12/13 (92) | —          |       |
| Raw food                          | 8/8 (100)    | 0/8 (0)   | 0/8 (0)  | 0/8 (0)  | 2/3 (67)  | 3/3 (100)       | 0/2 (0) | 0/2 (0)      | 0/2 (0)  | 2/2 (100)  | 0/2 (0)    | —           | —            | —         | —                            | 0/6 (0)          | 0/6 (0)    | 3/6 (50)   | 1/3 (33)                            | 0/3 (0)   | 2/3 (66)  | 1/3 (33)  | 3/3 (100)  | —          |       |
| Raw feed                          | 10/10 (100)  | 0/10 (0)  | 0/10 (0) | 0/10 (0) | 2/2 (100) | 2/2 (100)       | 0/1 (0) | —            | —        | —          | —          | —           | —            | —         | —                            | 0/5 (0)          | 0/5 (0)    | 5/5 (100)  | 4/5 (80)                            | 0/5 (0)   | 2/5 (40)  | 4/5 (80)  | 5/5 (100)  | —          |       |
| (Perk)                            |              |           |          |          |           |                 |         |              |          |            |            |             |              |           |                              |                  |            |            |                                     |           |           |           |            |            |       |

<sup>a</sup>The isolate contained substitution in code 83.

<sup>b</sup>The isolate contained substitution in codon 87.

<sup>c</sup>The five ciprofloxacin resistant isolates contained a single point mutation in the *gyrA* gene. Three of the isolates contained substitutions in codon 83 and two isolates in codon 87.

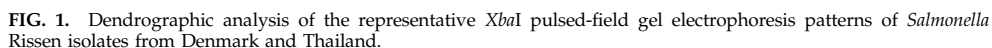
<sup>d</sup>The ceftiofur resistant isolates originating from a premature born child in Denmark contained the *bla*<sub>CTX-M-14</sub> gene.

<sup>e</sup>Isolated from both fresh meat, animal feed and deceased animal.

<sup>f</sup>One isolates from a premature child in Denmark accounted for resistance to AMP, CEF, POD, XNL, CHL, SPE, STR, SMX, TET, TMP.

<sup>g</sup>Travel histories were available for 9/23 Danish patients. Molecular characterization was performed on Resistant (R) and Intermediate (I) isolates.

AMP, ampicillin; CEF, cefalothin; FOD, cefodoxime; XNL, ceftiofur; CHL, chloramphenicol; SPE, spectinomycin; STR, streptomycin; XNL, ceftiofur; CHL, chloramphenicol; SPE, spectinomycin; STR, streptomycin; SMX, sulphamethoxazole; TET, tetracycline; TMP, trimethoprim.



food. With the exception of one isolate (1%) obtained from Spanish pork products, antimicrobial resistance within this cluster was limited to tetracycline.

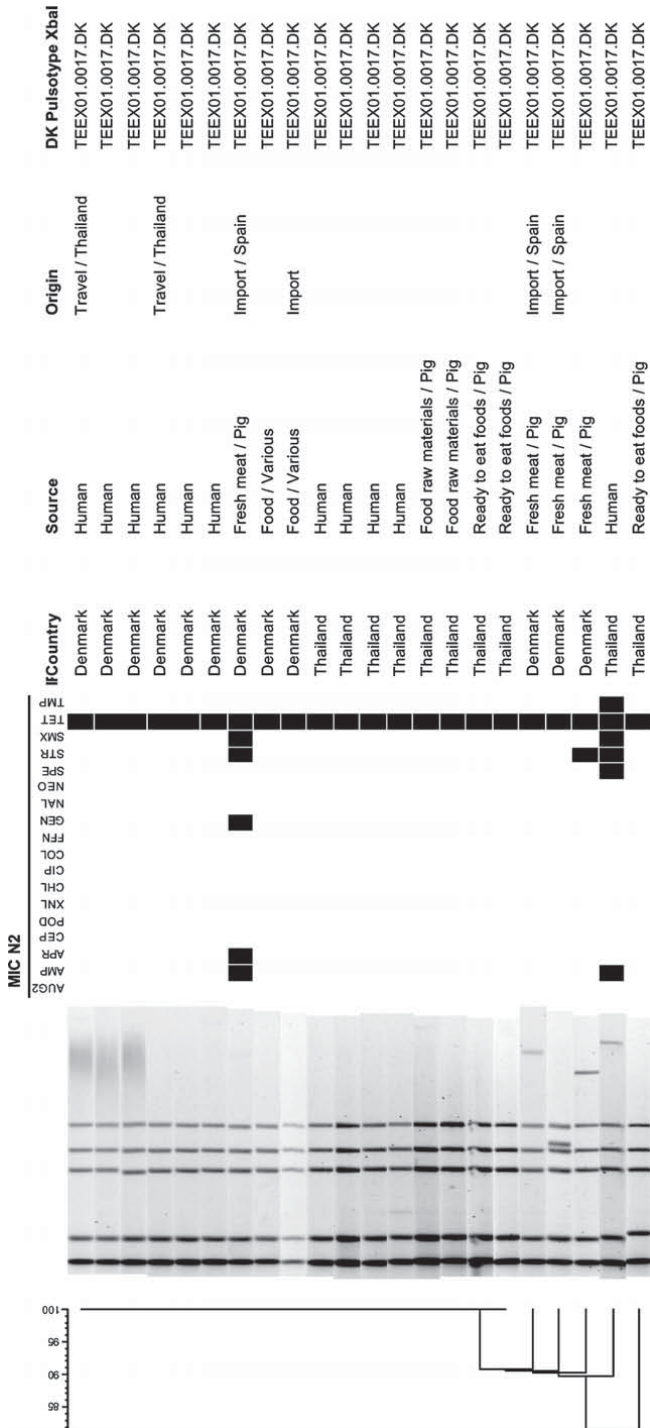


FIG. 2. Dendrographic analysis of *Salmonella* Rissen pulsed-field gel electrophoresis cluster TEEEX01.0017.dk following digestion with *BlnI*.

A similarity of approximately 82% was observed among PFGE cluster 1 strains. This cluster of isolates originated from Denmark and was pan-susceptible.

In PFGE cluster 6; the predominant pattern, TEEX01.0026.DK was shared by six isolates (5%): four from Thai patients (4%), one isolate (1%) from ready-to-eat food and one isolate (1%) from a Danish patient with a history of travel to Thailand prior to the infection. All of the isolates (5%) were resistant to AMP, CHL, NEO, SPE, STR, SMX, TET, and TMP (data not shown) (Fig. 1).

## Discussion

The majority of *Salmonella* Rissen isolates included in this study exhibited resistance to tetracycline and displayed limited resistance to other antimicrobials. A previous study from Thailand found almost all porcine isolates to be fully resistant to TET, STR, and SMX (Angkititrakul *et al.*, 2005). Thai isolates and isolates from Danish patients with a history of travel to Thailand were more likely to be resistant to AMP, CHL, STR, SMX, and TMP than isolates originating from Denmark.

Almost all ampicillin-resistant isolates contained a sequence similar to *bla*<sub>TEM-1b</sub>. *bla*<sub>TEM</sub> genes have also been widely found among *Salmonella* isolates in other studies (Gallardo *et al.*, 1999; Olesen *et al.*, 2004). Chloramphenicol resistance was mediated by the *catA1* or the *cmlA* gene, which has also been observed previously (Guerra *et al.*, 2002; Zhao *et al.*, 2007), but is also widespread among other gram-negative bacteria. Florfenicol resistance is normally encoded by the *flo* gene, which specifies nonenzymatic cross-resistance to both florfenicol and chloramphenicol. However, none of the florfenicol-resistant isolates tested contained the *flo* gene. It has previously been suggested that several of isolates may possess an unknown chromosomal *flo* gene (White *et al.*, 2000). Tetracycline resistance was primarily mediated by *tetA*. However, the *tetB* gene was identified in one turkey isolate and three of the eight Danish pig or pork isolates. *tetA* is commonly found on transposons such as Tn1721, this gene has been identified in many gram-negative bacteria including *Salmonella* (Frech and Schwarz, 2000).

Sulphonamide resistance in *Enterobacteriaceae* is typically mediated by the resistance genes *sulI* (encoded on a Class I integron), *sul2* (encoded on a nonconjugative plasmid), or *sul3* (putatively associated with both an integron system and a conjugative plasmid) (Radstrom *et al.*, 1991). *sul1*-, *sul2*-, and *sul3*-mediated resistance was identified among the sulphamethoxazole-resistant isolates.

The *aph(3'')-II* gene was identified in all neomycin-resistant isolates. This gene has previously been identified in several other species of gram-negative bacteria. Streptomycin resistance was encoded by *aadA*, *strA*, and *strB*. This genotype is also commonly observed in Denmark among streptomycin-resistant isolates of *Salmonella* Typhimurium (Madsen *et al.*, 2000).

Seven ciprofloxacin-resistant isolates contained a single mutation in *gyrA* at codons 83 (Ser → Phe) or 87 (Asp → Asn). Mutations at these positions, located within the quinolone resistance-determining region of *gyrA*, have been associated with ciprofloxacin resistance in several bacterial species, including high-level ciprofloxacin resistance in *Salmonella* spp. (Chiu *et al.*, 2002; Casin *et al.*, 2003; Ling *et al.*, 2003).

To our knowledge, this is the first study utilizing PFGE to demonstrate the molecular diversity of *Salmonella* Rissen. This appears to be a genetically diverse serotype, with 112 strains producing a total of 63 unique *XbaI* patterns (Fig. 1). The predominant pattern was TEEX01.0017.DK which consisted of 22 isolates and was shared between Thai patients, ready-to-eat food, raw food, Danish patients with and without a history of travel, and imported and Danish pig or pork. Several other patterns were shared by isolates from different sources which could support a link between the reservoirs.

The biological analysis strongly suggests that Thai patients acquire *Salmonella* Rissen infections by consumption of Thai-produced pig or pork products. These data also suggest that travel to Thailand and consumption of imported and Danish-produced pig or pork products are potential risk factors for infection with *Salmonella* Rissen infection among Danish patients. Previous studies have also indicated that Danish patients might acquire infections with *Salmonella* serotypes Corvallis or Schwarzengrund from Thailand through a combination of travelling

and the import of different food products (Archambault *et al.* 2006; Aarestrup *et al.* 2007). Source attribution analysis in Denmark has also shown the increased importance of imported food products as a source of *Salmonella* infections (Hald *et al.*, 2007). With increases in tourism and international trade, *Salmonella* subtypes which had been confined to specific regions may emerge elsewhere. These studies emphasise the need to view *Salmonella* epidemiology from a global perspective.

It should be noted that some degree of selection and statistical bias may have influenced the results of the present study. The majority of Thai isolates were collected between May and June 2004 and only isolates collected during this time period were selected for molecular characterization. In contrast, the Danish isolates were collected over a period of several years. Comparing isolates from different time periods may reduce the chances of finding clones with identical antibiogrammes or indistinguishable PFGE patterns. Additionally, the limited number of isolates included in the study might have influenced the statistical analysis of specific risk factors, particularly the consumption of imported pork. It should also be noted that travel histories could only be obtained for nine of the 23 Danish patients. No information regarding travel prior infection was available for 14 patients.

## Conclusion

This study suggest that travel to Thailand and the consumption of pig or pork products (Danish and imported) were risk factors for *Salmonella* Rissen infections among the Danish patients included in this study. This study also suggests that the Thai patients included in this study most likely became infected with *Salmonella* Rissen by the consumption of Thai pig or pork products. This study may have potential benefits for future *Salmonella* Rissen investigations and outbreak detection.

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## Article IV

### Emergence of multidrug-resistant *Salmonella* Concord infections in Europe and the United States in children adopted from Ethiopia, 2003-2007

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# Emergence of Multidrug-Resistant *Salmonella* Concord Infections in Europe and the United States in Children Adopted From Ethiopia, 2003–2007

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**Background:** Multidrug-resistant *Salmonella* serovar Concord infections have been reported from children adopted from Ethiopia. We interviewed patients, characterized the isolates, and gathered information about adoptions from Ethiopia to assess public health implications.

**Methods:** Information about *Salmonella* Concord cases and adoptions were provided from Austria, Denmark, England (and Wales), Ireland, the Netherlands and the United States. Patients from Denmark and the United States were interviewed to determine the orphanages of origin; orphanages in Ethiopia were visited. Isolates were subtyped by pulsed-field gel electrophoresis and antimicrobial susceptibility; specific antimicrobial resistance genes were characterized.

**Results:** *Salmonella* Concord was isolated from 78 persons from 2003 to 2007. Adoption status was known for 44 patients  $\leq 3$  years of age; 98% were adopted from Ethiopia. The children adopted from Ethiopia were from several orphanages; visited orphanages had poor hygiene and sanitation and frequent use of antimicrobial agents. The number of children adopted from Ethiopia in the participating countries increased 527% from 221 in 2003 to 1385 in 2007. Sixty-four *Salmonella* Concord isolates yielded 53 pulsed-field gel electrophoresis patterns including 6 patterns with  $>2$  indistinguishable isolates; one isolate from an Ethiopia adoptee. Antimicrobial susceptibility was performed on 43 isolates; 81% were multidrug-resistant ( $\geq 3$  agents). Multidrug-resistant isolates were from Ethiopian adoptees and were resistant to third and fourth generation cephalosporins and 14% had decreased susceptibility to ciprofloxacin.

**Conclusions:** Improved hygiene and sanitation and more appropriate use of antimicrobial agents are needed in orphanages in Ethiopia. Culturing of

stool specimens of children adopted from Ethiopia and appropriate hygiene may prevent further disease transmission.

**Key Words:** *Salmonella*, Ethiopia, adoptees, ESBL, multi-drug resistance

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*Salmonella enterica* is a common cause of human gastroenteritis worldwide.<sup>1–3</sup> Although most *Salmonella* infections are self-limiting, severe infections resulting in bacteremia, meningitis, and death may occur. Antimicrobial agents may be life-saving in severe infections. Third generation cephalosporins and fluoroquinolones are commonly used for the treatment of *Salmonella* infections in children and adults, respectively.<sup>4,5</sup> Infections caused by antimicrobial-resistant *Salmonella* are more likely to require hospitalization, and may result in more severe outcomes.<sup>6–8</sup>

In 2007, infections caused by *Salmonella* serovar Concord, a rare *Salmonella* serotype, were reported in several countries among children adopted from Ethiopia; the isolates from these infections were resistant to numerous antimicrobial agents including third generation cephalosporins.<sup>9,10</sup> To prevent further infections, we conducted a multinational investigation, in collaboration with the Ethiopia Ministry of Health, to determine the likely sources of the infections.

## PATIENTS AND METHODS

### Epidemiologic Information

Public health institutes in Europe and the United States which identified human *Salmonella* Concord infections in 2003 to 2007 were invited to participate in the study. Participating countries sent isolates to the National Food Institute (DTU-Food) in Denmark and provided information about patients including those  $\leq 3$  years of age. Adoption status and country of origin were provided if available. Patients, or parents of patients  $< 18$  years of age, in Denmark and the United States were interviewed to determine the orphanage of origin for adopted patients and if the patient had international travel before illness onset or used antimicrobial agents before specimen collection. Information about adoptions from Ethiopia was sought from national agencies in participating countries. In collaboration with the Ethiopian Nutrition and Health Research Institute, orphanages in Ethiopia were visited in February 2008.

### Laboratory

Isolates were serotyped at public health laboratories and confirmed at DTU-Food.<sup>11</sup> Isolates were subtyped by pulsed-field gel electrophoresis (PFGE) at state public health laboratories in the United States and DTU-Food according to the PulseNet protocol

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using *Xba* I digestion.<sup>12</sup> PFGE patterns were compared using BioNumerics 4.6 (Applied Maths, Sint-Martens-Latem, Belgium). Minimum inhibitory concentrations (MICs) to 25 antimicrobial agents were determined using Sensititre microbroth dilution.<sup>13</sup> CLSI interpretive criteria were used for amikacin, ampicillin, aztreonam, cefazolin, cefepime, cefpodoxime, ceftazidime, ceftriaxone, cefuroxime, cephalothin, chloramphenicol, ciprofloxacin, gentamicin, imipenem, nalidixic acid, sulfamethoxazole, tetracycline, and trimethoprim<sup>14–16</sup>; and DTU-Food-defined resistance breakpoints were used for apramycin (>16 mg/L), ceftiofur (>4 mg/L) (a third generation cephalosporin used in veterinary medicine), colistin (>8 mg/L), florfenicol (>16 mg/L) (a phenicol used in veterinary medicine), neomycin (>8 mg/L), spectinomycin (>64 mg/L), and streptomycin (>16 mg/L) ([http://www.crl-ar.eu/\\_pdf/monitoring\\_reports/Danmap%202006.pdf](http://www.crl-ar.eu/_pdf/monitoring_reports/Danmap%202006.pdf)). Decreased susceptibility to ceftriaxone and ciprofloxacin was defined as an MIC  $\geq 2$  mg/L and MIC  $\geq 0.125$  mg/L, respectively. Resistance to  $\geq 3$  antimicrobial agents of different classes was defined as multidrug-resistant.

Multidrug-resistant strains were further characterized using a polymerase chain reaction (PCR) assay with primers specific for 8 antimicrobial resistance genes<sup>13</sup> (Table, Supplemental Digital Content 1, <http://links.lww.com/INF/A144>). PCR products were purified (GFX PCR DNA kit Amersham Biosciences), and submitted to Macrogen Inc. for sequencing. Sequence analysis and alignment was performed using Vectors NTI suite 9 (InforMax, Inc.). Resulting nucleotide sequences were compared with se-

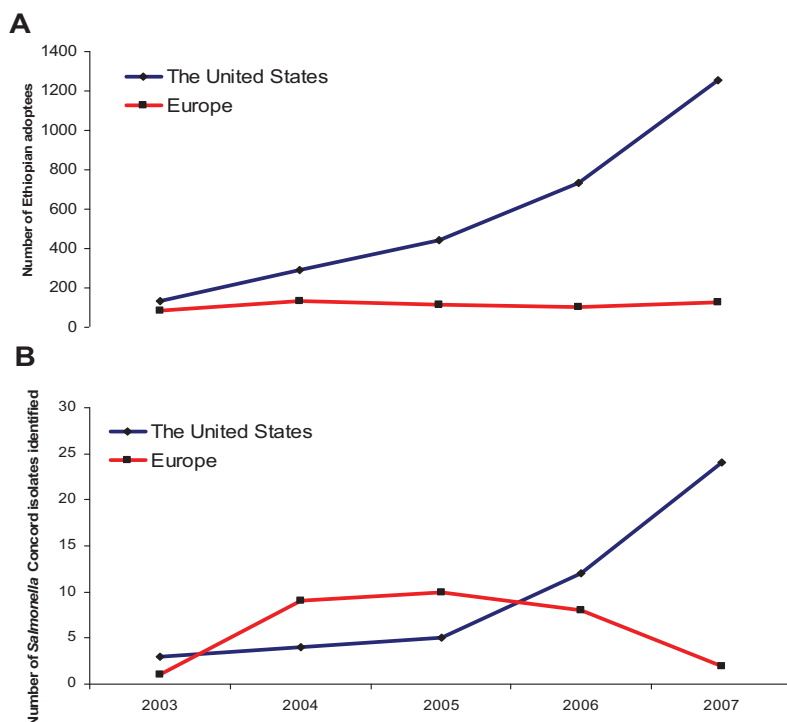
quences obtained from GenBank (available at: <http://www.lahey.org/studies/webt.html>). Conjugation of selected multidrug-resistant isolates was performed using previously described methods.<sup>17,18</sup> Transconjugation was verified by PCR using primers specific for *bla*<sub>CTX-M-15</sub> and *bla*<sub>SHV-12</sub>. Plasmid analysis was performed on selected transconjugants and their respective donors by S1-nuclease digestion and PFGE.

## Role of Funding Source

Neither of the grants for this study had any involvement in design, collection of isolates, analysis, interpretation of data, preparation of the article or decision where to submit the study for publication.

## RESULTS

Public health institutes in Austria, Denmark, England (and Wales), Ireland, the Netherlands and the United States reported 78 cases of laboratory-confirmed *Salmonella* Concord infections from 2003 to 2007. In the United States, *Salmonella* Concord was isolated from 48 persons; 3 in 2003, 4 in 2004, 5 in 2005, 12 in 2006, and 24 in 2007. In the 5 participating European countries, *Salmonella* Concord was isolated 30 persons; 1 in 2003, 9 in 2004, 10 in 2005, 8 in 2006 and 2 in 2007 (Fig. 1). During the study period, *Salmonella* Concord was isolated from 12 persons in Austria, 3 in Denmark, 9 in England (and Wales), 2 in Ireland, and 4 in the Netherlands. Gender were known for 67 patients; 41 (61%) were female. Age was known for 75 patients. The median age was



**FIGURE 1.** Number of children adopted from Ethiopia (A) and number of reported laboratory-confirmed cases of *Salmonella* serotype Concord (B) per year in participating countries in Europe\* and the United States, 2003–2007. \*No adoption data were available for Austria. References: <http://www.adoptionsnaevnet.dk/>; [http://travel.state.gov/family/adoption/notices/notices\\_473.html](http://travel.state.gov/family/adoption/notices/notices_473.html); <http://www.adoptie.nl/>; <http://www.adoptionboard.ie/>; <http://www.dfes.gov.uk/intercountryadoption/general.shtml>.

12 months (range: 2 months–76 years); 56 (75%) were  $\leq 3$  years of age and 11 (15%) were  $> 18$  years of age. Adoption status was known for 44 (79%) of the patients  $\leq 3$  years of age; of these, 43 (98%) were adopted. The patient who was  $\leq 3$  years of age and was not adopted was a sibling of a child adopted from Ethiopia. All 43 adopted children were from Ethiopia except for 1 child who was adopted from an unspecified African country. Of the 43 adopted children, 10 adopted children were brought to Austria and 33 to the other participating countries (29 to the United States, 3 to Denmark, and 1 to England). Six (54%) patients  $> 18$  years of age were female; of these, 2 were mothers of children adopted from Ethiopia.

We interviewed patients or parents for 31 (61%) of the 51 patients in Denmark and the United States. Among the 25 interviewed patients  $\leq 3$  years of age, 24 (96%) were adopted from Ethiopia. For the children adopted from Ethiopia, the median time in one or more orphanages in Ethiopia was 3 months (range: 1–6.5 months). Stool specimens which yielded *Salmonella* Concord from the children adopted from Ethiopia were collected an average of 32 days following adoption (range: 2–185 days). Six (25%) of the children adopted from Ethiopia were asymptomatic at the time of adoption and specimen collection; 1 asymptomatic child had a stool specimen cultured because of an ill sibling, and 5 asymptomatic children had stool specimens cultured resulting from recommendations by his or her pediatrician. Eighteen (75%) of the children adopted from Ethiopia were symptomatic at the time of adoption; all had diarrhea, 7 (39%) had fever; 4 (22%) had bloody diarrhea, and 3 (17%) were hospitalized. Median duration of illness was 11 days (range: 5–90 days). One child received antimicrobial agents after illness onset and before specimen collection. Information regarding the adoption agency in Ethiopia was reported for 18 (75%) of the adopted children; the children were adopted from 8 different orphanages in Addis Abba, Ethiopia.

A total of 3419 children were adopted from Ethiopia from 2003 to 2007 and brought to countries participating in this study (no adoption information was available from Austria); during this 5-year period, the number of children adopted from Ethiopia increased 527% from 221 adoptions in 2003 to 1385 in 2007. Of the 2852 children adopted from Ethiopia and brought to the United States, 1987 (70%) occurred in the last 2 years. Of the 567 children adopted from Ethiopia and brought to 1 of the 4 European countries with adoption information participating in this study, 233 (41%) occurred in the last 2 years. During the study period, 188 children adopted from Ethiopia were brought to Denmark, 14 to England (and Wales), 66 to Ireland, and 299 to the Netherlands (Fig. 1).

Two orphanages in Ethiopia from where at least 3 patients were adopted were visited. Children at the orphanages were most commonly abandoned at police stations shortly after birth. Family or medical history before arrival at the orphanage was seldom known. Children typically stayed at the orphanages for at least 3 months before being adopted or sent to another agency. Poor hygiene and sanitation was observed at the orphanages. Cases of dehydration and diarrhea were reported among the children in the orphanages. According to physicians at the orphanages, young children in the orphanages were typically treated for diarrhea with ceftriaxone, gentamicin and sulfamethoxazole, or with trimethoprim and sulfamethoxazole; older children received ciprofloxacin.

### Laboratory

*Salmonella* Concord isolates from 64 (82%) of the 78 patients were subtyped by PFGE. Fifty-three unique *Xba*I PFGE patterns were observed (Fig., Supplemental Digital Content 2, <http://links.lww.com/INF/A145>). There were 6 PFGE patterns

with  $\geq 2$  indistinguishable isolates. The pattern with the most indistinguishable isolates included those from 7 children of which at least 5 isolates were from children adopted from Ethiopia. Each of the remaining 5 patterns with  $\geq 2$  indistinguishable isolates included at least 1 isolate from a child adopted from Ethiopia including 1 pattern with indistinguishable isolates from a child adopted from Ethiopia and his adopted mother.

Isolates were available for antimicrobial susceptibility testing for 43 (55%) of the 78 patients; 8 (19%) were susceptible to all agents and 35 (81%) were multidrug-resistant. Travel history was known for 4 of the patients infected with pansusceptible *Salmonella* Concord. None reported associations with Ethiopia but all were adults who traveled to Kenya before illness onset; one adult also traveled to South Africa, Zambia, and Malawi. Travel or adoption status was known for 30 of the 35 patients infected with multidrug-resistant isolates. All were either from or associated with a child adopted from Ethiopia. All multidrug-resistant isolates were resistant to ampicillin, aztreonam, cefazolin, cefepime, cefpodoxime, ceftazidime, ceftiofur, cefuroxime, cephalothin chloramphenicol, streptomycin, sulfamethoxazole, and trimethoprim. All multidrug-resistant isolates also had decreased susceptibility to ceftriaxone; 34 (97%) were ceftriaxone-resistant. Of the multidrug-resistant isolates, 34 (97%) were resistant to gentamicin, 24 (69%) were resistant to tetracycline, and 6 (14%) showed decreased susceptibility to ciprofloxacin.

At least 1 isolate was available for antimicrobial susceptibility testing from 3 of the 6 PFGE patterns with  $\geq 2$  indistinguishable isolates; each of these available isolates was multidrug-resistant and was from a child adopted from Ethiopia.

All 35 multidrug-resistant isolates harbored a *bla*<sub>TEM</sub> gene and *bla*<sub>CTX</sub> gene; sequence analysis of the PCR products showed 100% identity to *bla*<sub>TEM-1b</sub> and *bla*<sub>CTX-M-15</sub>, respectively. Of the multidrug-resistant isolates, 13 (37%) also harbored the *bla*<sub>SHV</sub> gene; sequence analysis revealed 100% identity to *bla*<sub>SHV-12</sub>. Nine multidrug-resistant isolates were selected for conjugation studies. Five transconjugants were successfully recovered yielding the same susceptibility pattern as the donors. After digestion with S1 enzyme and PFGE, a plasmid of approximately 380 kb. was observed. Two isolates yielded transconjugants with less resistance than the donors (resulted in limited resistance to ampicillin, cephalothin, cefpodoxime, and ceftiofur). After digestion with S1 and PFGE, a single plasmid of approximately 80 kb was observed in these 2 isolates. PCR confirmed the presence of the genes *bla*<sub>CTX</sub> and the *bla*<sub>SHV</sub> in all transconjugants.

The 6 multidrug isolates with decreased susceptibility to ciprofloxacin were characterized. Three isolates were indistinguishable by PFGE and contained the plasmid mediated quinolone resistance gene *qnrB*; these isolates were isolated from children adopted from Ethiopia and brought to the United States. Two isolates with different PFGE patterns contained the quinolone resistance gene *qnrA* gene; these isolates were isolated from children adopted from Ethiopia and brought to Austria. The remaining isolate had a single base substitution in the *gyrA* gene at codon 83 ([TCC {Ser}  $\rightarrow$  TTC {Phe}]); this isolate was from a 1-year old child in the United Kingdom with an unknown adoption history.

### DISCUSSION

In this multinational study, we demonstrate that multidrug-resistant *Salmonella* Concord infections are common among children adopted from Ethiopia. We found that from 2003 to 2007, at least 33 (1.0%) of the 3419 children adopted from Ethiopia and brought to the United States and 4 European countries had a laboratory-confirmed *Salmonella* Concord infection. In the United

States alone the number was 24 cases of 2852 (0.8%) Ethiopian adoptees. Most of these infected children were symptomatic, some with severe symptoms. Since only a fraction of *Salmonella* infections are laboratory-confirmed, these data suggest a remarkably high incidence of *Salmonella* infection among children in orphanages in Ethiopia. It is not known how long this *Salmonella* strain has been present in these orphanages, but the diversity of PFGE patterns (no indication of temporal evolution among the patterns) among the children adopted from Ethiopia and the adoption of infected children from at least 8 orphanages in Ethiopia indicates an endemic problem in Ethiopian orphanages. The increasing isolation of this strain in the United States and Europe likely reflects that increasing frequency of adoption of children from Ethiopia. Ethiopia was the fourth most common country of origin for adoptions in Denmark and the United States in 2007 following China, Vietnam and South Africa in Denmark (Available at: <http://www.adoptionsnaevnet.dk>), and China, Guatemala and Russia in the United States (Available at: [http://travel.state.gov/family/adoption/stats/stats\\_451.html](http://travel.state.gov/family/adoption/stats/stats_451.html)).

The highly resistant nature of the *Salmonella* Concord isolates from children adopted from Ethiopia makes antimicrobial treatment difficult. Although antimicrobial agents are not necessary for the treatment of most *Salmonella* infections, antimicrobial treatment can be life-saving in severe infections.<sup>4,5</sup> All of the isolates from children adopted from Ethiopia were resistant to 19 antimicrobial agents including all antimicrobial agents commonly used to treat *Salmonella* infections in children. Furthermore, some of the isolates from children adopted from Ethiopia had decreased susceptibility to ciprofloxacin; treatment of such infections with fluoroquinolones is not advised because such treatment has been associated with treatment failures.<sup>19,20</sup>

The highly resistant isolates from children adopted from Ethiopia illuminates the need for more appropriate use of antimicrobial agents in orphanages in Ethiopia. The empiric treatment of children with diarrhea at the orphanages with a combination of ceftriaxone, gentamicin, and sulfamethoxazole is particularly worrisome. It is not known if other countries have similar endemic *Salmonella* problems in orphanages but the transmission of multidrug-resistant *Salmonella* has been reported in orphanages in other countries in Africa; in a similar study of multidrug-resistant *Salmonella* Babelsberg and *Salmonella* Enteritidis infections in France among children adopted from Mali, the highly resistant nature of the isolates was thought to be due to the heavy use of antimicrobial agents in orphanages in Mali.<sup>21</sup> Preventing further infections in orphanages in Ethiopia and elsewhere should focus on improvements in hygiene and sanitation. The highly resistant nature of *Salmonella* Concord in the orphanages in Ethiopia demonstrates the difficulties in controlling such infections using antimicrobial agents. Treatment of children with diarrhea should focus on supportive care particularly rehydration. Antimicrobial agents should be reserved for treatment of patients at risk for serious infections or with systemic symptoms.

This study provides useful information for parents adopting children from Ethiopia and perhaps elsewhere. The American Academy of Pediatrics recommends that a stool specimen be collected from all adopted children and cultured for bacterial pathogens including *Salmonella*.<sup>22–24</sup> Adherence to this recommendation identified *Salmonella* Concord infections in several asymptomatic children adopted from Ethiopia. The utility of this recommendation was highlighted in this study since we identified several instances in which family members were infected with *Salmonella* Concord which was apparently introduced into the family from an adopted child. Furthermore, considering the alarmingly high frequency of antimicrobial resistance among the *Sal-*

*monella* Concord isolates from adopted children in this study including resistance to third and fourth generation cephalosporins and ciprofloxacin, it may be useful to test *Salmonella* isolates isolated from adopted children for antimicrobial susceptibility.

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Table 1. Oligonucleotide primer sequences used for the amplification of the various resistance genes.

| Resistant gene            | Sequence  | Annealing temp.(°C) | Amplicon size (bp) |
|---------------------------|---|---------------------|--------------------|
| <i>bla</i> <sub>CTX</sub> | 5'-CCGTTTCCSCTATTACAAACCG-3'<br>5'-GATCCGCGTGATACCACTTCA-3'           | 65                  | 354                |
| <i>bla</i> <sub>CTX</sub> | 5'-CCATGGTTAAAAAATCACTGCG-3'<br>5'-TGGGTRAARTARGTSACCAGAAYSAGCGG-3'   | 60                  | 805                |
| <i>bla</i> <sub>TEM</sub> | 5'-ACCAATGCTTAATCAGTGAG-3'<br>5'-GCGGAACCCCTATTG-3'                   | 55                  | 1017               |
| <i>bla</i> <sub>SHV</sub> | 5'-TTATCTCCCTGTAGCCACC-3'<br>5'-GATTTGCTGATTCGCTCGG-3'                | 60                  | 797                |
| <i>qmrA</i>               | 5'-GGATGCCAGTTTCGAGGA-3'<br>5'-TGCCAGGCACAGATCTTG-3'                  | 59                  | 492                |
| <i>qmrB</i>               | 5'-ATGACGCCATTACTGTATAA-3'<br>5'-GATCGCAATGTGAAAGTTT-3'               | 53                  | 562                |
| <i>qmrS</i>               | 5'-CGACGTGCTAACTTGCGTGATA-3'<br>5'-TACCCAGTGCTTCGAGAAATCAG-3'         | 57                  | 538                |
| <i>aac(6')Ib</i>          | 5'-TTGCGATGCTCTATGAGTGGCTA-3'<br>5'-CTCGAAATGCCTGGCGTGTTT-3'          | 55                  | 482                |
| <i>gyrA</i>               | 5'-TAC CGT CAT AGT TAT CCA CGA-3'<br>5'-GTA CTT TAC GCC ATG AAC GT-3' | 60                  | 313                |





## Article V

### **Molecular characterization of extended spectrum cephalosporinases (ESC) producing *Salmonella Choleraesuis* from patients in Thailand and Denmark**

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Molecular characterization of extended spectrum cephalosporinases (ESC) producing *Salmonella* Choleraesuis from patients in Thailand and Denmark

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Running title: Extended spectrum cephalosporinases (ESC) producing *Salmonella* Choleraesuis  
in Thailand and Denmark

Key words: *Salmonella* Choleraesuis, bacteremia, extended spectrum cephalosporinases,  
antimicrobial resistance, PFGE.

## ABSTRACT

The objective of this study was to characterize extended spectrum cephalosporinases (ESC) producing isolates of *Salmonella* serovar Choleraesuis recovered from patients in Thailand and Denmark.

Twenty-four isolates were included in the study, 13 of which were blood culture isolates. Twenty-three isolates were recovered from Thai patients in 2003, 2007, or 2008 and one isolate was recovered from a Danish traveller to Thailand. ESC production was confirmed by minimum inhibitory concentration testing. Micro-array and plasmid profiling (replicon typing and RFLP) were used to characterize the genetic mechanisms of antimicrobial resistance in the ESC producing isolates. PFGE was used to compare isolates resistant to third generation cephalosporins with susceptible isolates from Thailand during the same period.

MIC determination, micro-array, PCR, plasmid profiling and replicon typing revealed the presence of multi-drug resistant isolates harboring either *bla*<sub>CMY-2</sub> containing *incA/C* or *bla*<sub>CTX-M-14</sub> containing *incFIIA* / *incFrepB* located on plasmids ranging in size from 75–200 kb. The RFLP and replicon typing clustered the isolates into four distinct groups. PFGE revealed 16 unique patterns and five clusters; each cluster contained two to three isolates. The isolate from the Danish patient was indistinguishable from two Thai clinical isolates.

This study revealed the emergence of the *bla*<sub>CTX-M-14</sub> gene among several clones of *Salmonella* serovar Choleraesuis. Numerous plasmids were identified containing up to two different ESC genes and four distinct replicons. A “travel associated” spread was confirmed. The findings represent a serious threat to public health for the Thai people and tourists.

## INTRODUCTION

*Salmonella enterica* is a common cause of human gastroenteritis and bacteremia worldwide (15, 29) and a wide variety of animals, particularly food animals, has been identified as reservoirs for non-Typhi *Salmonella* (11, 19, 20). Although approximately 2,600 serovars of *Salmonella enterica* have been identified, most human infections are caused by a limited number of serovars and in general these infections are self-limiting. Some *Salmonella* serovars including *Salmonella* Choleraesuis (swine) and *Salmonella* Dublin (cattle) which are adapted to a specific animal hosts, have a propensity to cause extra-intestinal infections in humans. When compared to other serovars of non-Typhi *Salmonella*, infections with these serovars are associated with higher rates of bacteremia, meningitis, and mortality (4, 5, 21). For patients with severe salmonellosis, antimicrobial chemotherapy may be life-saving. Due to increasing prevalence of fluorquinolone resistance third generation cephalosporins are increasingly used for the treatment of *Salmonella* infections in humans (14, 18, 22) and these compounds have been designated as critically important for human health by the World Health Organization (10).

We recently reported that human infections with *Salmonella* serovar Choleraesuis in Thailand increased from 1.5% in 1993 to 9.2% in 2007 (16). The group of people at highest risk for these infections was those between 6–40 years of age in the Central region of Thailand (16). A 2007 study of *Salmonella* serovar Choleraesuis isolates from Thailand observed an increasing resistance to both third generation cephalosporins and fluoroquinolones. Fifty-four isolates obtained between 2003 and 2005 were tested, of which 30% were found to be resistant to third generation cephalosporins (22).

To date, only two reports, both from Taiwan, have described mechanisms for third generation cephalosporin resistance in *Salmonella* serovar Choleraesuis. The first report was published in 2004 with the discovery of *bla*<sub>CMY-2</sub> AmpC  $\beta$ -lactamase gene located on a 140 kb F-like plasmid (6). The following year, the same authors detected *bla*<sub>CTX-M-3</sub> in a *Salmonella* serovar Choleraesuis isolate from a patient admitted to a university hospital (28). In 2007, a massive increase of flouroquinolone and ceftriaxone resistant *Salmonella* serovar Choleraesuis was described in Thailand (22).

In Taiwan, the usage of antimicrobials in veterinary medicine and as growth promoter in animal feed may have promoted the emerging of the resistance (5). Likewise, in Thailand, the third generation cephalosporin ceftiofur is used extensively in the swine production (22).

The objectives of the present study were to determine the genetic diversity and antimicrobial resistance of extended spectrum cephalosporinases (ESC) producing *Salmonella* serovar Choleraesuis isolates from patients in Thailand and Denmark. Additionally, isolates of *Salmonella* serovar Choleraesuis from Thailand which were resistant to third generation cephalosporins were compared with susceptible isolates using PFGE.

## METHODS

### *Bacterial isolates*

A total of 24 isolates were included in this study. Twenty-three isolates were recovered in Thailand and one ESC producing isolate was recovered in 2008 at Aalborg Hospital, Aarhus University Hospital, Denmark. The WHO National *Salmonella* and *Shigella* Center in Bangkok receives all presumptive isolates of *Salmonella* spp. from all diagnostic laboratories throughout Thailand. In 2003, as part of another study, the National Food Institute, Technical University of

Denmark (DTU-Food) received 82 isolates of *Salmonella* serovar Choleraesuis which were recovered from Thai patients. (1). In 2008, this collection was screened for the presence of ESC producing isolates and two ESC producing strains, both isolated in 2003, were identified. DTU-Food received 12 isolates of *Salmonella* serovar Choleraesuis in 2008, ten isolates were ESC producers isolated in 2008 from Thai patients at the Regional Medical Sciences Center in Samutsongkhram, Thailand.

In addition, to assess the genetic diversity of *Salmonella* serovar Choleraesuis in Thailand, nine isolates from Thailand which were susceptible to third generation cephalosporins were included in the study. These susceptible isolates were isolated from patients in Bangkok and were randomly selected from the collection.

### *Serotyping*

The isolates were serotyped using slide agglutination in the country of origin.

O and H antigens were characterized by agglutination with hyperimmune sera (S & A reagents lab, Ltd, Bangkok, Thailand and Statens Serum Institute, Copenhagen, Denmark) and serotype was assigned according to the Kauffmann-White scheme (13).

### *Antimicrobial susceptibility testing*

Minimum inhibitory concentration testing of the 13 ESC producing isolates was performed at the DTU-Food using previously described methods (17). Results were primarily interpreted using current European Committee on Antimicrobial Susceptibility Testing (EUCAST) ([www.eucast.org](http://www.eucast.org)) and European Food Safety Authority (EFSA) epidemiologic break points (26). Due to the absence of some break points in the EUCAST system, exceptions were made for the interpretation of cefepime and ceftriaxone where Clinical and Laboratory Standards Institute

guidelines and clinical breakpoints were utilised (7, 8, 9). Quality control using *E. coli* ATCC 25922 was conducted according to CLSI.

#### *Microarray*

Detection of gene-groups associated with the antimicrobial resistance phenotypes was carried out using miniaturized microarrays (Identibac Amr-ve Array tubes, New Haw, Addlestone, Surrey, UK) containing probes for most relevant Gram-negative antimicrobial gene groups (Identibac). Analysis was performed as described by the manufacturer on the 13 ESC producing isolates.

#### *Detection of genes conferring resistance to extended spectrum cephalosporinases*

PCR amplification and sequencing of the *bla*<sub>CTX-M-9</sub> group, *bla*<sub>TEM</sub> and *bla*<sub>CMY-2</sub> genes were performed on the 13 isolates using methods described previously (17, 25).

Amplicons produced were selected for sequencing. Prior to sequencing, the amplicons were purified using the GFX™ PCR DNA kit (GE Healthcare, Chalfont St. Giles, UK) following the protocol of the manufacturer. The DNA was shipped to Macrogen Inc., Seoul, Korea for sequencing using the same primers as in the PCR analysis. Sequence analysis and alignment was performed using Vectors NTI suite 9 (InforMax Inc., Bethesda, Maryland, US) software. The resulting nucleotide sequences were compared to sequences obtained from the GenBank database (<http://www.lahey.org/studies/webt.html>).

#### *Plasmid characterization*

Plasmid DNA was extracted using QIAGEN Plasmid Mini Kit (Qiagen, Hilden, Germany). The plasmid DNA was transferred into electrocompetent *E. coli* DH10B cells and subjected to S1 nuclease PFGE as described below to ensure that only one plasmid had been transferred into the



competent cells as well as to estimate the approximate size of the plasmids carrying the ESC phenotype. The electroporation was followed by selection of transformants on BHI agar supplemented with cefotaxime (2 µg/ml). The presence of the plasmid in the transformants was confirmed by PCR detection of relevant *bla* genes as described above. Additionally, testing was performed to determine if any non-ESC resistance determinants co-transferred with the ESC plasmids. The 13 transformants were analysed by PCR for all relevant resistance genes based on the results of the Array tube analysis described above. Transformants were further subjected to replicon PCR and plasmid purification followed by RFLP.

#### *Replicon typing*

Plasmids within transformants were replicon typed as described previously (2).

#### *Transferability of bla genes by conjugation*

Plate-mating experiments were performed with transformants as donors and plasmid-free, rifampicin and nalidixic acid resistant *E. coli* MT102RN as recipients (2, 25). The strains were grown to both late exponential as well as stationary phase, mixed (1:1) and incubated on solid blood agar at 37°C for 18 h. Transconjugants were selected on BHI medium supplemented with 50 µg/ml rifampicin, 32 µg/ml nalidixic acid, and 2 µg/ml cefotaxime.

#### *S1 digestion for plasmid size determination*

PFGE with S1 nuclease (Promega) digestion of whole genomic DNA performed as described below was used to estimate sizes of larger plasmids. Following pre-incubation for 10 min. in 1:10 diluted S1 buffer, 2 mm slices of PFGE plugs made from cultures with an OD<sub>620</sub> of 0.6 were

digested with 5 U of S1 (Promega, Madison, US) for 45 min. at 37°C. The slices were post-incubated on ice for 10 min in 200 µL of ice-cold TE-buffer (10:1), loaded on the gel and run on a CHEF-DRIII device (Bio-Rad, Hercules, USA) with a pulse time of 6.8 s – 38.4 s at 6 V/cm for 19 h. *S. Braenderup* H9812 digested with *Xba*I was used as size marker .

#### *Pulsed-field gel electrophoresis*

All 24 isolates included this study were analyzed for genetic relatedness by PFGE using *Xba*I according to the CDC PulseNet protocol (24). The electrophoresis was performed with a CHEF DR III System (Bio-Rad Laboratories, Hercules, CA, USA) using 1% SeaKem agarose in 0.5× Tris-borate-EDTA at 180 V. Running conditions consisted of one pulse time of 2.2 - 63.8 s for 22 h at 6 V/cm on a 120 deg. angle in 14°C TBE buffer. Comparison of the PFGE profiles was performed by using Bionumerics software version 4.6 (Applied Maths, Sint-Martens-Latem, Belgium) and the dice correlation for band matching with a 0.9 % position tolerance and an optimization at 0.9 % using both *Xba*I.

## **RESULTS**

#### *Epidemiological data of the Thai patients*

Twelve of the ESC producing isolates from Thai patients were obtained from blood samples; 11 of the isolates were obtained in Ratchaburi Province and one was from Bangkok. The 12 isolates were obtained from 10 patients with two patients each having two positive blood cultures. All samples were collected between April and May or between August and October. The patients were unevenly distributed by gender, with eight isolates obtained from males and two from females. The age of one patient was unknown. However, the age of the remaining nine patients

ranged from 17 to 58 years with a median of 34 years. Data on occupation of the patients were not collected.

#### *Epidemiological investigations of the Danish patient*

A healthy 37-year old Danish male was on assignment to an industrial company in Bangkok from July 27 to August 14 in 2008. He resided in a five star international hotel at Sukhumvit Road in the center of Bangkok and worked full time at the Navanakorn Industrial Estate Zone 3 situated 45 km outside of Bangkok. Meals were primarily served in the hotel and at the work place. Typical Thai food (soup and rice dishes) was daily consumed and included either fish or minced pork.

One week before the return to Denmark the patient contracted diarrhoea with an acute onset, but without blood. The patient was febrile up to 38.8°C with dizziness, cephalgia, and mild muscle pain. Concurrently, the patient noticed flexor paresis of the interphalangeal joint of the left thumb accompanied by hypesthesia in the C6 dermatome. Except for paresis and hypesthesia the symptoms abated in a few days and the diarrhoea responded to loperamide. However, symptoms recurred several times and the patient was admitted to a Danish hospital on September 10<sup>th</sup> with a weight loss of 11 kg. A blood culture obtained by admission revealed *Salmonella* serovar Choleraesuis. Fecal cultures during admission were negative.

Empirical antimicrobial treatment included ciprofloxacin and pivmecillinam. Treatment with meropenem was considered based on the multi-drug resistant phenotype of the isolate, but the patient felt well and he declined. Eight months later the patient was in good health though the flexor paresis persisted.

#### *Antimicrobial resistance*

All of the 13 ESC producing isolates were multi-drug resistant and exhibited resistance to at least 13 of the tested antimicrobials (eight antimicrobial drug classes) (Figure 1). Resistance was not detected to apramycin (only approved for veterinary use), colistin, imipenem, meropenem and trimethoprim. All isolates were resistant to ampicillin, cefalothin, cefazolin, cefotaxime, cefpodoxime, ceftiofur, nalidixic acid, sulphamethoxazole and tetracycline. Twelve isolates (92%) were resistant to chloramphenicol; four (31%) of these strains also were resistant to florfenicol (only approved for veterinary use). Resistance to ciprofloxacin was observed in twelve (92%) isolates, one isolate had a MIC just below the break point of ciprofloxacin. Eleven (85%), four (31%), four (31%), and four (31%) of the isolates were resistant to the ceftriaxone, ceftazidime, ceftipime, and cefoxitin, respectively (Figure 1). Four isolates (31%) were also resistant to amoxicillin and clavulanic acid and another six isolates (46%) to gentamicin. Only two isolates (15%) were resistant to neomycin whereas eleven (85%) and ten (77%) isolates were resistant to spectinomycin and streptomycin, respectively.

#### *Identification of resistance genes*

Based on Array tube analysis and subsequent sequencing of PCR-generated DNA fragments, eight of the 13 ESC producing isolates harbored the extended spectrum  $\beta$ -lactamase gene group *bla*<sub>CTX-M-9</sub>, the chloramphenicol resistance acetyltransferase gene *cmIA*, the sulphonamide resistance gene *sul3*, the aminoglycoside resistance gene *aadA1*, and the tetracycline resistance gene *tetB*. In addition, five (SH2867/08, SH2868/08, SH2871/08, SH2872/08, 08-120226) of the eight isolates also harbored the integrase gene *int1*.

Of the remaining five isolates, four contained the *floR* gene conferring resistance to florfenicol, the sulphonamide resistance gene *sul2*, the integrase gene *int1* encoding an intergron, the aminoglycoside resistance genes *strA* and *strB*, the plasmidic ampC gene *bla*<sub>CMY</sub>, and the

tetracycline gen *tetA*. In addition, one (SH508/03) of the four isolates also harbored the *tetB* and *sul1* genes. Furthermore, three (SH2870/08, SH2874/08, SH508/03) of the four isolates contained also the aminoglycoside resistance gen *aadA1*.

One isolate (SH1208/03) was resistant to sulphamethoxazole and tetracycline containing the *sul1* and *tetB* genes, respectively. In addition, this isolate harbored the extended spectrum  $\beta$  - lactamase gen group *bla*<sub>CTX-M-9</sub> and the *bla*<sub>TEM</sub> gen.

The isolates which produced amplicons to *bla*<sub>CTX-M-9</sub> group, *bla*<sub>CMY</sub> and *bla*<sub>TEM</sub> were sequenced and showed 100% similarity to strains in the Genbank encoding for *bla*<sub>CTX-M-14</sub>, *bla*<sub>CMY-2</sub> and *bla*<sub>TEM-1b</sub>, respectively.

#### *Plasmid characterization by S1 nuclease PFGE, replicon typing and RFLP*

Approximate plasmid sizes were given in Figure 2 and ranged from approximately 75 kb to 200 kb. Plasmid profiling by RFLP separated the plasmids into three distinct clusters (I, II and III in Figure 2). Furthermore, one plasmid profile (from strain SH1208/03) was not associated to any of the three main groups. The plasmid with *bla*<sub>CMY-2</sub> genes was associated to cluster I, while plasmids harboring the *bla*<sub>CTX-M-14</sub> gene belonged to the two remaining clusters as well as the plasmid out of line with the three clusters (Figure 2). Replicon typing identified the *incA/C* replicon in RFLP cluster I, the *incF<sub>rep</sub>* in cluster II and *incFIIA* in cluster III, while the plasmid from SH1208/03 was untypable.

#### *Co-transfer of non-ESC antimicrobial susceptibility phenotypes*

The PCR results revealed that only one (SH2862/08) of the eight ESC producing isolates which harbored the extended spectrum  $\beta$ -lactamase gen group *bla*<sub>CTX-M-14</sub> successfully transferred non-

ESC resistance determinants. In addition to *bla*<sub>CTX-M-14</sub>, the transformant contained the *sul3*, the *aadA1* and the *tetB* genes.

The four transformants containing plasmid ampC gen *bla*<sub>CMY-2</sub> seemed to harbor many of the non-ESC resistance determinants. All of the four transformants contained the *floR*, the *sul2*, the *strA* / *strB* and *tetA* genes. In addition, one (SH508/03) of the four transformants also contained the *sul1* gene and three transformants (SH2870/08, SH2874/08, SH508/03) contained the *aadA1* gene.

The transformant of the one isolate (SH1208/03) which harbored the *bla*<sub>CTX-M-14</sub> also contained the *bla*<sub>TEM</sub> gen.

#### *Conjugation experiments*

By conjugation experiments we found the ESC phenotype readily transferable from wild type strains carrying plasmids belonging to RFLP cluster I and cluster II as well as from isolate SH 1208/03. However, the four strains carrying the *incFIIA* type plasmids of RFLP cluster III did not succeed.

#### *PFGE typing*

The 24 *Salmonella* serovar Choleraesuis isolates from 22 patients were subtyped by PFGE. Sixteen unique *XbaI* PFGE patterns were observed (Figure 1). There were five distinct PFGE clusters with  $\geq 2$  indistinguishable isolates. Three clusters contained ESC producing isolates of which two included two indistinguishable isolates from different Thai patients (SH2858/08, SH2867/08 and SH2870/08, SH2874/08). A second cluster contained three isolates with indistinguishable patterns, two isolates from one Thai patient (SH2871-08, SH2872-08) (different susceptibility profiles) and the isolate (08-120226) from the Danish traveller to Thailand. The

two remaining clusters only contained isolates susceptible to third generation cephalosporins of which one included isolates from both 2003 and 2008.

## DISCUSSION

This study provides the first description of *bla*<sub>CTX-M-14</sub> in *Salmonella* serovar Choleraesuis isolates and is the first reported isolation of an ESC producing *Salmonella* serovar Choleraesuis in an international traveller who recovered with sequelae from his infection. In addition, these data provide evidence that ESC producing isolates have emerged in Thailand on several plasmids and in several clusters of *S. Choleraesuis*.

Characterization of the antimicrobial resistance genes indicates some similarity between isolates harboring either *bla*<sub>CTX-M-14</sub> or *bla*<sub>CMY-2</sub>. The data from the plasmid characterization, conjugation, replicon typing and RFLP also suggested that these are not highly clonal strains and further grouped the isolates into four distinct replicon clusters. Based on the data of the unknown replicon, one could speculate if the plasmid of isolate SH1208/03 was the ancestor to the other isolates harboring the *bla*<sub>CTX-M-14</sub> gene and simply evolved rather than spread to other strains. All of the analyses indicate multiple clones and multiple plasmids being responsible for the resistance to extended spectrum cephalosporinases producing *Salmonella* serovar Choleraesuis obtained from patients in Thailand and Denmark.

Several studies have described plasmids carrying *bla*<sub>CMY-2</sub> containing the *incA/C* replicon along with other resistance genes. A recent Canadian study investigated 38 *E. coli* isolates where all of the isolates harbored a plasmid carrying *bla*<sub>CMY-2</sub> containing the *incA/C* replicon (24). A similar association between *bla*<sub>CTX-M14</sub> and *incFII* has been described previously. Geraldine Marcade *et al* found that the great majority of genes encoding *bla*<sub>CTX-M-14</sub> and *bla*<sub>CTX-M-15</sub> were carried by

IncF replicons. Of 15 *E. coli* isolates harboring *bla*<sub>CTX-M-14</sub> eight of them contained the replicon IncFII (23).

PFGE patterns revealed a high degree of clonal diversity among the 24 isolates. ESC producing and non-ESC producing isolates were generally interspersed although some rare clusters comprised solely resistant or susceptible isolates. This indicates that multiple clones are circulating in the population and that isolates resistant to third generation cephalosporins most likely developed due to a long standing selection pressure from the use of these compounds in a veterinary reservoir rather than a recent spread of a single clone.

*Salmonella* serovar Choleraesuis has been eradicated from the primary production of swine in Denmark and many other industrialised countries. The isolate from the Danish traveller shared the an identical PFGE pattern with an isolate from a Thai patient infected in the autumn of 2008. The isolates were resistant to the same antimicrobials and harbored the same resistance genes with exception of two additional resistance traits in the isolate from the Danish patient, namely resistance to amoxicillin + clavulanic acid and gentamicin. We have no explanation for this discrepancy because the Danish patient did receive only symptomatic therapy prior to admission to the hospital in Denmark.

The Danish case was remarkable for neurologic symptoms localized to the left hand which coincided with diarrhoeal illness and persisting for at least 9 months. There was no evidence of focal infection, but the similarity with mononeuropathy in association with typhoid fever may indicate a common pathogenesis (12).



Thailand is a popular tourist destination for Europeans. Thus, in 2008 149.570 Danes visited Thailand (<http://www.tourism.go.th/>). During the same year, 3,022 confirmed cases of *Salmonella* infections in humans were reported to Statens Serum Institute, Denmark. Of these, 706 (23.3%) were confirmed as travel associated and 95 (13.4%) cases were linked to travelling to Thailand (Data not published). These numbers might be underestimated and probably should be multiplied with 10 -20 times (30). Taking this underestimation into consideration 0.6% of the Danes visiting Thailand might bring back a *Salmonella* infection based on these data.

The infections with *Salmonella* serovar Choleraesuis has recently increased in Thailand and the emergence of ESC producing *Salmonella* serovar Choleraesuis makes this problem even more serious (16). We therefore urge the Thai authorities to take action in order to prevent and control the spread of this serovar among animals and the human population. Targeted interventions can benefit swine farmers by reducing losses and possible export restrictions. These interventions can also reduce the high costs of hospitalization associated with treatment of invasive *Salmonella* serovar Choleraesuis infections which include the necessity to use carbapenems which are antibiotics of last resort. We recommend enforcing a strict policy on the usage of antimicrobials in food animals and a ban on the usage of third generation cephalosporins as growth promoter.

## CONCLUSION

This study provides for the first time a description of *bla*<sub>CTX-M-14</sub> found in *Salmonella* serovar Choleraesuis isolates and documents a case of bacteremia with an ESC producing *Salmonella* serovar Choleraesuis acquired by a Danish traveller during a stay in Bangkok. The data suggest that ESC producing isolates have emerged in Thailand on several plasmids and in multiple clones of *Salmonella* serovar Choleraesuis.

We found two genes and four replicons responsible for the resistance to third and fourth generation cephalosporins present in the isolates from both 2003 and 2008. In addition, the isolates exhibit a huge diversity among the molecular patterns indicating a variable population despite having similar resistance patterns and genes.

The Thai authorities should initiate immediate actions to control and prevent infections with this invasive serovar for the benefit of the Thai people and tourists travelling to Thailand. The first step could be specific serovar targeted intervention and limitations in the usage of third generation cephalosporins as growth promoters in the primary production of pigs.

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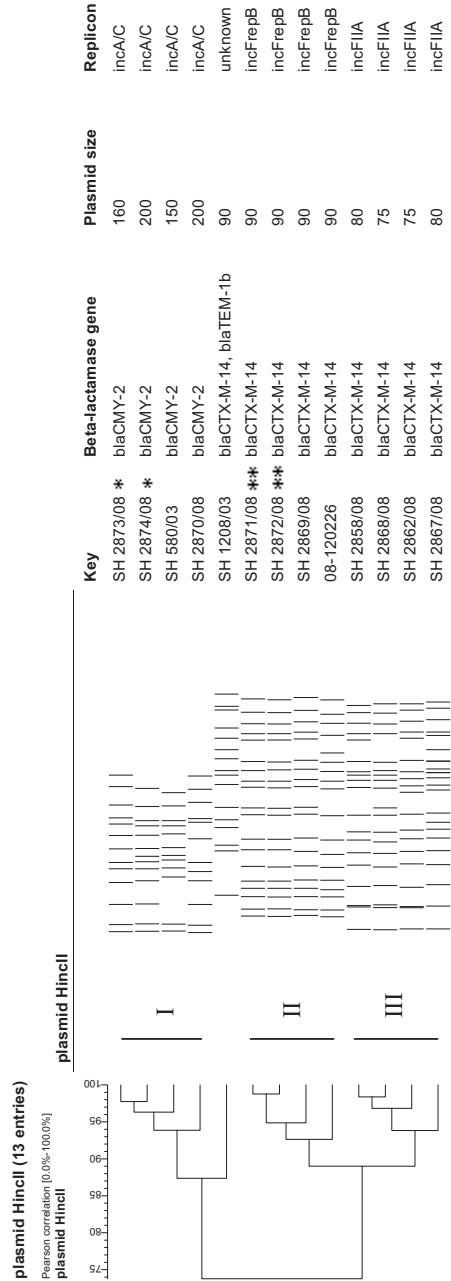
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Figure 2. Dendrographic analysis of plasmid replicons subjected to RFLP of *Salmonella* Choleraesuis transformants originating from Thai and Danish patients.



Plasmid sizes are in kb., \*: same patient, \*\*: same patient.

## Article VI

### **Risk factors and epidemiology of the ten most common *Salmonella* serovars from patients in Thailand; 2002 - 2007.**

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## Risk Factors and Epidemiology of the Ten Most Common *Salmonella* Serovars from Patients in Thailand: 2002–2007

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### Abstract

We conducted a retrospective observational study to assess epidemiological trends and risk factors associated with the 10 most common *Salmonella* serovars isolated from humans in Thailand between 2002 and 2007. A total of 11,656 *Salmonella* isolates covering all 6 years were included in the study. The top 10 *Salmonella* serovars identified during the course of this study were Enteritidis, Stanley, Weltevreden, Rissen, I [1],4,[5],12:i:–, Choleraesuis, Anatum, Typhimurium, Corvallis, and Panama, which accounted for 8108 (69.6%) of the isolates. Most isolates were from patients <5 years (33%), were isolated during June (13%), and were recovered from stool (82%) and from patients in Bangkok (27%). Statistical analysis revealed that *S. Enteritidis* and *S. Choleraesuis* were recovered from blood with a higher frequency than other nontyphoidal serovars. While both serovars tended to be isolated from patients >5 years; *S. Choleraesuis* was recovered with a higher frequency from patients in Bangkok and the central region, whereas *S. Enteritidis* was recovered predominantly from patients in the southern region. This study also indicates a shift in prevalence of the most common *Salmonella* serovars responsible for human infections in Thailand compared to previous studies. Notably, there was an increase in human infections with *S. Stanley*, *S. Corvallis*, and *S. Choleraesuis*, three serovars that have previously been associated with swine, and a decrease in infections due to *S. Weltevreden* and *S. Anatum*. The study also revealed differences in the epidemiology among the different serovars, suggesting that serovar-specific interventions are needed. We recommend initiating targeted interventions for the two serovars associated with a high odds ratio for submitted blood samples, *S. Enteritidis* and *S. Choleraesuis*. The authors also recommend additional epidemiologic studies to investigate the observed increase in swine associated serovars (*S. Stanley*, *S. Corvallis*, and *S. Choleraesuis*) and determine interventions to reduce the burden of disease from these serovars.

### Introduction

*SALMONELLA ENTERICA* IS A COMMON CAUSE of human gastroenteritis and bacteremia and a wide variety of animals, particularly food animals, have been identified as reservoirs for nontyphoidal *Salmonella* (Coyle *et al.*, 1988; Humphrey *et al.*, 1988; Humphrey, 2000). A limited number of the 2579 currently recognized serovars of *Salmonella* account for the vast majority of human infections. In developed countries, *S. Typhimurium* and *S. Enteritidis* are the most common causes of human salmonellosis, but other serovars have been reported to be more prevalent in specific regions (Humphrey, 2000; Herikstad *et al.*, 2002; Aarestrup *et al.*, 2003; Bangtrakulnonth *et al.*, 2004; Galanis *et al.*, 2006).

Knowledge of the prevalence and epidemiology of different serovars in specific regions may facilitate the recognition and control of new and emerging pathogens. Previous studies have shown that *Salmonella* serovars Choleraesuis, Dublin, Virchow, Enteritidis, and Panama tend to cause invasive disease and are associated with higher mortality rates. These studies have reported mortality rates of 1.8% and 3.0% for *S. Choleraesuis* and *S. Dublin*, respectively, compared with 0.5% for all nontyphoidal *Salmonella* and 0.6% for *S. Typhimurium* (Helms *et al.*, 2002; Chiu *et al.*, 2004; Jones *et al.*, 2008). The explanations for this observed increased virulence have not been fully elucidated and are most likely multifactorial.

The following retrospective observational cross-sectional study was conducted to elucidate the epidemiological trends

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of the 10 most common *Salmonella* serovars isolated from humans in Thailand between 2002 and 2007. Additionally, an analysis of risk factors for human salmonellosis in Thailand was performed. Risk factor analysis for infection with 1 of the 10 most common nontyphoidal *Salmonella* serovars was compared to other nontyphoidal *Salmonella* serovars in the study. *Salmonella* serovars Typhi, Paratyphi A, and Paratyphi B were not included in the study based on the relatively low prevalence in Thailand (2.1%) (Annual Report of Confirmed *Salmonella* and *Shigella* in Thailand, 2006). The following variables were included in the analysis: age group, season, sex, specimen type, and geographical region.

The results of this study provide insight into the epidemiology and the specific factors responsible for human salmonellosis among patients in Thailand. These data may assist with targeted interventions to control infections by invasive *Salmonella* serovars.

## Methods

### Data source

The World Health Organization (WHO) National *Salmonella* and *Shigella* Centre in Bangkok receives all presumptive isolates of *Salmonella* from all public health laboratories in Thailand. Confirmatory identification is performed at the WHO National *Salmonella* and *Shigella* Centre using approved internationally recognized procedures. Following confirmation, isolates are serotyped by slide agglutination as previously described (Bangtrakulnonth *et al.*, 2004).

For each isolate, the following clinical and epidemiological data are electronically recorded in Microsoft Excel 2000 spreadsheets (Microsoft Corporation, Redmond, WA): journal number, laboratory number, date, given name, surname, sex, age including age group, origin, geographical zone, origin of sample, specimen, serogroup, and serotype. There is no indication in the data source if only one or more samples have been submitted per patient.

### Data set

The data set contained of a total of 11,656 *Salmonella* isolates with complete information covering the years from 2002 to 2007 (Table 1). The dependent variables were the 10 most common serovars in Thailand. Odds ratio of being infected with each of the 10 serovars were calculated as being infected with serovar number 1 compared to the rest of the serovars in the data set, the odds ratio of being infected with serovar number 2 compared to the rest of the serovars in the data set, etc.

The qualitative variables—age group, season, and region—were aggregated into fewer levels due to the number of degrees of freedom, which caused problems when running the algorithm. Originally, age groups were given in intervals of 5 years but were aggregated into only five levels: 0–5, 6–20, 21–40, 41–60 and >60 years. Similarly, season and regions were aggregated from months into four seasonal periods (winter, spring, summer, and autumn) and from 13 zones into five regions (central, northeast, southern, northern, and Bangkok). Many of the strains were isolated from various parts of the body and from various materials, thus only specimens originating from blood and fecal samples (rectal swabs or stool) were included in the analysis (Table 1).

### Statistical analysis

The statistical package SAS version 9.1.3 (SAS Institute Inc., Cary, NC) was used to perform the logistic analysis. A preselection of independent variables was initiated using univariable analysis and all independent variables with a *p*-value of <0.05 was included in logistic analysis. A full model was fitted for each serotype including all independent variables. Backward selection was performed using *p*-values. The criteria for keeping variables in the model was *p*-values <0.05. The full model and the reduced model were compared using the log likelihood ratio test to test the contribution of the independent variables in the final model. The final models contained several different independent variables. To understand the meaning of the models, the odds ratio of being infected with 1 of the 10 most common serovars compared to the remaining other nontyphoidal *Salmonella* serovars was calculated for each level of the independent variables (Tables 2 and 3). Biological meaningful two-factor interactions were not analyzed as they were beyond the scope of this article.

## Results

### Descriptive data

A total of 11,656 isolates collected from Thai patients with salmonellosis between 2002 and 2007 were included in this study. The 10 most common *Salmonella* serovars in this study were *S. Enteritidis* (*n* = 1517 [13%]); *S. Stanley* (*n* = 1292 [11%]); *S. Weltevreden* (*n* = 1055 [9%]); *S. Rissen* (*n* = 969 [8%]); *S. I* [1,4,5,12:i:–] (*n* = 690 [6%]); *S. Choleraesuis* (*n* = 681 [6%]); *S. Anatum* (*n* = 551 [5%]); *S. Typhimurium* (*n* = 540 isolates [5%]); *S. Corvallis* (*n* = 476 isolates [4%]); and *S. Panama* (*n* = 337 isolates [3%]), which accounted for 8108 (69.6%) of the isolates (Table 1). We have not received any information to suggest that these data are biased or disproportionate due to the occurrence of a local or regional outbreak.

The proportion of the nontyphoidal *Salmonella* serovars decreased from 49.6% (*n* = 1629) in 1993 to 24.4% (*n* = 1981) in 2007. The proportion of *S. Anatum*, which peaked at 10.1% (*n* = 412) in 2000, decreased to 0.3% (*n* = 7) in 2006. Similarly, by 2006, the proportion of *S. Weltevreden*, had decreased 13 years in a row to 7.3% (*n* = 151) and continued to decline in 2007, representing 6.6% (*n* = 130) of cases. During this same period, increases in *S. Choleraesuis* were observed. The proportion of *S. Choleraesuis* isolates increased from 2.8% (*n* = 55) in 2002 to 9.2% (*n* = 190) in 2006. This study also retrospectively identified the emergence in 2003 of *S. Corvallis*, a serovar not recorded in Thailand since 1993. From 2003 to 2007, the level of *S. Corvallis* remained fairly constant, representing approximately 4.5% (*n* = 45) of cases. The proportion of *S. Rissen* remained relatively constant from 1997 to 2004, ranging from 5.9% (*n* = 46) of cases in 1998 to 9.6% (*n* = 81) of cases in 2004. The proportion of *S. Panama* decreased in 2002 from 6.7% (*n* = 130) of cases to 2.4% (*n* = 33) of cases in 2003 and remained at this level through 2007. The proportion of *S. Stanley* increased since 1993 (1.9%; *n* = 64) to a maximum level in 2003 (10.1%; *n* = 141). While decreases in the proportions of *S. Enteritidis* and *S. I* [1,4,5,12:i:–] were observed in 2004 and 2005 compared to previous years, subsequent increases were observed in 2007 with *S. Enteritidis*

TABLE 1. DESCRIPTIVE DATA OF THE VARIABLE AND LEVELS USED FOR ANALYZING THE SIGNIFICANT FACTORS CAUSING INFECTIONS AMONG THAI PATIENTS WITH THE TOP 10 MOST COMMON *SALMONELLA* SEROVARS IN 2002-2007

| Variables            | Levels                        | Overall<br>N (%) | S.<br>Enteritidis<br>N (%) | S.<br>Stanley<br>N (%) | S.<br>Waltveden<br>N (%) | S.<br>Rissen<br>N (%) | S.<br>I [11,14,15,12:i:-<br>N (%) | S.<br>Choleraesuis<br>N (%) | S.<br>Anatum<br>N (%) | S.<br>Typhimurium<br>N (%) | S.<br>Corvallis<br>N (%) | S.<br>Panama<br>N (%) | Others<br>N (%) |
|----------------------|-------------------------------|------------------|----------------------------|------------------------|--------------------------|-----------------------|-----------------------------------|-----------------------------|-----------------------|----------------------------|--------------------------|-----------------------|-----------------|
| Age group<br>(years) | 0-5                           | 3830 (32.9)      | 210 (13.8)                 | 664 (51.4)             | 274 (26.0)               | 368 (38.0)            | 265 (38.4)                        | 78 (11.5)                   | 119 (21.6)            | 188 (34.8)                 | 175 (36.8)               | 184 (54.6)            | 1305 (36.8)     |
|                      | 6-20                          | 1283 (11.1)      | 134 (10.2)                 | 131 (10.2)             | 171 (13.3)               | 101 (7.9)             | 44 (3.4)                          | 54 (4.2)                    | 75 (5.85)             | 53 (4.1)                   | 56 (4.4)                 | 27 (3.1)              | 437 (12.4)      |
|                      | 21-40                         | 3021 (25.9)      | 636 (21.1)                 | 174 (5.8)              | 237 (7.9)                | 222 (7.4)             | 208 (6.9)                         | 329 (10.9)                  | 168 (5.6)             | 134 (4.4)                  | 94 (3.1)                 | 51 (1.7)              | 768 (25.4)      |
|                      | 41-60                         | 1871 (16.1)      | 302 (19.9)                 | 156 (12.1)             | 185 (17.4)               | 145 (15.0)            | 106 (15.4)                        | 124 (18.2)                  | 99 (18.0)             | 91 (16.9)                  | 71 (14.9)                | 39 (11.6)             | 553 (15.6)      |
|                      | >60                           | 1651 (14.2)      | 235 (15.9)                 | 167 (12.9)             | 188 (17.8)               | 133 (13.7)            | 67 (9.7)                          | 96 (14.1)                   | 90 (16.3)             | 74 (13.7)                  | 80 (16.8)                | 36 (10.7)             | 485 (13.7)      |
| Season               | Winter                        | 2551 (21.9)      | 379 (25.0)                 | 305 (23.6)             | 236 (22.4)               | 234 (24.2)            | 147 (21.3)                        | 152 (22.3)                  | 124 (22.5)            | 100 (18.5)                 | 86 (18.1)                | 71 (21.1)             | 717 (20.2)      |
|                      | (Dec-Feb.)                    |                  |                            |                        |                          |                       |                                   |                             |                       |                            |                          |                       |                 |
|                      | Spring                        | 2871 (24.6)      | 334 (22.0)                 | 322 (24.9)             | 232 (22.0)               | 229 (23.6)            | 233 (33.8)                        | 172 (25.3)                  | 114 (20.7)            | 148 (27.4)                 | 129 (27.1)               | 118 (35.0)            | 840 (23.7)      |
|                      | (Mar-May)                     |                  |                            |                        |                          |                       |                                   |                             |                       |                            |                          |                       |                 |
|                      | Summer                        | 3485 (29.9)      | 423 (27.9)                 | 358 (27.7)             | 343 (32.5)               | 299 (30.9)            | 154 (22.3)                        | 159 (23.4)                  | 204 (37.0)            | 180 (33.3)                 | 140 (29.4)               | 94 (27.9)             | 1130 (31.9)     |
| Sex                  | (Jun-Aug.)                    |                  |                            |                        |                          |                       |                                   |                             |                       |                            |                          |                       |                 |
|                      | Autumn                        | 2750 (23.6)      | 381 (25.1)                 | 307 (23.8)             | 244 (23.1)               | 207 (21.4)            | 156 (22.6)                        | 198 (29.1)                  | 109 (19.8)            | 112 (20.7)                 | 121 (25.4)               | 54 (16.0)             | 861 (24.3)      |
|                      | (Sep-Nov.)                    |                  |                            |                        |                          |                       |                                   |                             |                       |                            |                          |                       |                 |
|                      | Female                        | 5591 (48.0)      | 680 (44.8)                 | 608 (47.1)             | 565 (53.6)               | 486 (50.2)            | 295 (42.8)                        | 267 (39.2)                  | 295 (53.5)            | 272 (50.4)                 | 237 (49.8)               | 154 (45.7)            | 1732 (48.8)     |
|                      | Male                          | 6065 (52.0)      | 837 (55.2)                 | 684 (52.9)             | 490 (46.5)               | 483 (49.9)            | 395 (57.3)                        | 414 (60.8)                  | 256 (46.5)            | 268 (49.6)                 | 239 (50.2)               | 183 (54.3)            | 1816 (51.2)     |
| Specimen             | Feces (stool,<br>rectal swab) | 9578 (82.2)      | 594 (39.2)                 | 1254 (97.1)            | 1037 (98.3)              | 954 (98.5)            | 496 (71.9)                        | 86 (12.6)                   | 546 (99.1)            | 426 (78.9)                 | 458 (96.2)               | 313 (92.9)            | 3414 (96.2)     |
|                      | Blood                         | 2078 (17.8)      | 923 (60.8)                 | 38 (2.9)               | 18 (1.7)                 | 15 (1.6)              | 194 (28.1)                        | 595 (87.4)                  | 5 (0.9)               | 114 (21.1)                 | 18 (3.8)                 | 24 (7.1)              | 134 (3.8)       |
|                      | Central                       | 4057 (34.8)      | 484 (31.9)                 | 411 (31.8)             | 345 (32.7)               | 314 (32.4)            | 197 (28.6)                        | 463 (68.0)                  | 203 (36.8)            | 152 (28.2)                 | 192 (40.3)               | 127 (37.7)            | 1169 (33.0)     |
|                      | (zone 1, 2, 3, 4)             |                  |                            |                        |                          |                       |                                   |                             |                       |                            |                          |                       |                 |
|                      | Northeast                     | 1228 (10.5)      | 146 (9.6)                  | 172 (13.3)             | 124 (11.8)               | 94 (9.7)              | 64 (9.3)                          | 32 (4.7)                    | 78 (14.2)             | 39 (7.2)                   | 28 (5.9)                 | 19 (5.6)              | 432 (12.2)      |
| Region               | (zone 5, 6, 7)                |                  |                            |                        |                          |                       |                                   |                             |                       |                            |                          |                       |                 |
|                      | Southern                      | 1450 (12.4)      | 277 (18.3)                 | 112 (8.7)              | 267 (25.3)               | 65 (6.7)              | 89 (12.9)                         | 11 (1.6)                    | 25 (4.5)              | 94 (17.4)                  | 42 (8.8)                 | 29 (8.6)              | 439 (12.4)      |
|                      | (zone 11, 12)                 |                  |                            |                        |                          |                       |                                   |                             |                       |                            |                          |                       |                 |
|                      | Northern                      | 1802 (15.5)      | 299 (19.7)                 | 181 (14.0)             | 156 (14.8)               | 164 (16.9)            | 145 (21.0)                        | 72 (10.6)                   | 86 (15.6)             | 103 (19.1)                 | 66 (13.9)                | 33 (9.8)              | 497 (14.0)      |
|                      | (zone 8, 9, 10)               |                  |                            |                        |                          |                       |                                   |                             |                       |                            |                          |                       |                 |
| Bangkok<br>(zone 13) | 3119 (26.8)                   | 311 (20.5)       | 416 (32.2)                 | 163 (15.5)             | 332 (34.3)               | 195 (28.3)            | 103 (15.1)                        | 159 (28.9)                  | 152 (28.2)            | 148 (31.1)                 | 129 (38.3)               | 1011 (28.5)           |                 |
| Overall N            |                               | 11656            | 1517                       | 1292                   | 1055                     | 969                   | 690                               | 681                         | 551                   | 540                        | 476                      | 337                   | 3548            |

TABLE 2. MULTIVARIABLE ANALYSIS OF THE SIGNIFICANT SINGLE FACTORS IN THE TOP 10 NONTYPHOIDAL *SALMONELLA* SEROVARS CAUSING SALMONELLOSIS AMONG THE THAI PATIENTS COMPARED TO OTHER NONTYPHOIDAL *SALMONELLA* SEROVARS

| Variables         | Levels       | S. Enteritidis |            |              | S. Stanley |           |              | S. Weltevreden |           |              | S. Rissen |           |              | S. I 11], 4, [5], 12;i:- |           |              |
|-------------------|--------------|----------------|------------|--------------|------------|-----------|--------------|----------------|-----------|--------------|-----------|-----------|--------------|--------------------------|-----------|--------------|
|                   |              | OR             | (95 % CI)  | p-value (LR) | OR         | (95 % CI) | p-value (LR) | OR             | (95 % CI) | p-value (LR) | OR        | (95 % CI) | p-value (LR) | OR                       | (95 % CI) | p-value (LR) |
| Age group (years) | 0-5          | 1.00           |            | <0.0001      | 1.00       |           | <0.0001      | 1.00           |           | <0.0001      | 1.00      |           | <0.0001      | 1.00                     |           | <0.0001      |
|                   | 6-20         | 1.58           | 1.24-2.02  |              | 0.56       | 0.46-0.69 |              | 1.91           | 1.55-2.35 |              | 0.44      | 0.32-0.61 |              | 0.44                     | 0.32-0.61 |              |
|                   | 21-40        | 2.18           | 1.81-2.61  |              | 0.37       | 0.31-0.44 |              | 1.40           | 1.16-1.69 |              | 0.78      | 0.64-0.96 |              | 0.78                     | 0.64-0.96 |              |
|                   | 41-60        | 1.86           | 1.51-2.29  |              | 0.50       | 0.42-0.61 |              | 1.67           | 1.36-2.04 |              | 0.67      | 0.53-0.86 |              | 0.67                     | 0.53-0.86 |              |
|                   | >60          | 1.79           | 1.44-2.21  |              | 0.60       | 0.50-0.72 |              | 1.86           | 1.52-2.27 |              | 0.49      | 0.37-0.65 |              | 0.49                     | 0.37-0.65 |              |
| Region            | Bangkok      | 1.00           |            | <0.0001      | 1.00       |           | <0.0001      | 1.00           |           | <0.0001      | 1.00      |           | <0.0001      | 1.00                     |           | <0.0001      |
|                   | Central      | 0.83           | 0.70-0.99  |              | 0.90       | 0.78-1.05 |              | 1.71           | 1.40-2.08 |              | 0.77      | 0.65-0.91 |              | 0.77                     | 0.62-0.94 |              |
|                   | Northeastern | 1.11           | 0.91-1.35  |              | 1.05       | 0.87-1.28 |              | 1.75           | 1.38-2.20 |              | 0.97      | 0.80-1.19 |              | 1.33                     | 1.05-1.67 |              |
|                   | Northern     | 1.11           | 0.88-1.41  |              | 1.36       | 1.11-1.66 |              | 1.78           | 1.39-2.28 |              | 0.69      | 0.54-0.88 |              | 0.97                     | 0.72-1.30 |              |
|                   | Southern     | 2.45           | 2.01-3.00  |              | 0.61       | 0.49-0.76 |              | 3.87           | 3.14-4.77 |              | 0.39      | 0.30-0.51 |              | 1.10                     | 0.84-1.43 |              |
| Specimen          | Feces        | 1.00           |            | <0.0001      | 1.00       |           | <0.0001      | 1.00           |           | <0.0001      | 1.00      |           | <0.0001      | 1.00                     |           | <0.0001      |
|                   | Blood        | 11.12          | 9.77-12.66 |              | 0.16       | 0.12-0.22 |              | 0.07           | 0.04-0.11 |              | 0.06      | 0.04-0.11 |              | 2.01                     | 1.67-2.43 |              |
| Season            | Summer       | 1.00           |            | 0.0027       |            |           |              |                |           |              |           |           |              | 1.00                     |           | 0.0027       |
|                   | Spring       | 0.89           | 0.75-1.06  |              |            |           |              |                |           |              |           |           |              | 1.84                     | 1.49-2.28 |              |
|                   | Autumn       | 1.12           | 0.95-1.33  |              |            |           |              |                |           |              |           |           |              | 1.25                     | 0.99-1.58 |              |
|                   | Winter       | 1.22           | 1.03-1.45  |              |            |           |              |                |           |              |           |           |              | 1.26                     | 1.00-1.60 |              |

TABLE 3. MULTIVARIABLE ANALYSIS OF THE SIGNIFICANT SINGLE FACTORS IN THE TOP 10 NONTYPHOIDAL *SALMONELLA* SEROVARS CAUSING SALMONELLOSIS AMONG THE THAI PATIENTS COMPARED TO OTHER NONTYPHOIDAL *SALMONELLA* SEROVARS

| Variables         | Levels       | S. <i>Choleraesuis</i> |             |              | S. <i>Anatum</i> |           |              | S. <i>Typhimurium</i> |          |              | S. <i>Coralis</i> |          |              | S. <i>Panama</i> |           |              |
|-------------------|--------------|------------------------|-------------|--------------|------------------|-----------|--------------|-----------------------|----------|--------------|-------------------|----------|--------------|------------------|-----------|--------------|
|                   |              | OR                     | (95% CI)    | p-value (LR) | OR               | (95% CI)  | p-value (LR) | OR                    | (95% CI) | p-value (LR) | OR                | (95% CI) | p-value (LR) | OR               | (95% CI)  | p-value (LR) |
| Age group (years) | 0-5          | 1.00                   |             | 0.0140       |                  |           | <0.0001      |                       |          |              |                   |          | <0.0001      |                  |           |              |
|                   | 6-20         | 1.63                   | 1.08-2.46   |              | 1.00             |           |              |                       |          |              |                   |          |              | 1.00             |           |              |
|                   | 21-40        | 1.51                   | 1.12-2.04   |              | 2.19             | 1.62-2.96 |              |                       |          |              |                   |          |              | 0.50             | 0.33-0.75 |              |
|                   | 41-60        | 1.14                   | 0.81-1.60   |              | 2.62             | 2.04-3.35 |              |                       |          |              |                   |          |              | 0.44             | 0.32-0.61 |              |
|                   | >60          | 1.17                   | 0.82-1.67   |              | 2.19             | 1.66-2.90 |              |                       |          |              |                   |          |              | 0.52             | 0.36-0.75 |              |
| Region            |              |                        |             | <0.0001      |                  |           |              |                       |          |              |                   |          |              | 0.53             | 0.37-0.77 |              |
|                   | Bangkok      | 1.00                   |             |              | 1.00             |           |              |                       |          |              |                   |          |              | 1.00             |           |              |
|                   | Central      | 3.29                   | 2.57-4.22   |              | 0.93             | 0.74-1.15 |              |                       |          |              |                   |          |              | 0.88             | 0.68-1.14 |              |
|                   | Northeastern | 0.60                   | 0.43-0.84   |              | 0.87             | 0.66-1.15 |              |                       |          |              |                   |          |              | 0.58             | 0.39-0.86 |              |
|                   | Northern     | 0.77                   | 0.50-1.20   |              | 0.97             | 0.73-1.29 |              |                       |          |              |                   |          |              | 0.46             | 0.28-0.76 |              |
| Specimen          | Southern     | 0.19                   | 0.10-0.36   |              | 0.26             | 0.17-0.40 |              |                       |          |              |                   |          |              | 0.51             | 0.34-0.77 |              |
|                   |              |                        |             | <0.0001      |                  |           |              |                       |          |              |                   |          |              | 0.60             | 0.42-0.85 |              |
|                   |              |                        |             |              |                  |           |              | 0.0381                |          |              |                   |          |              |                  |           | <0.0001      |
| Season            | Feces        | 1.00                   |             |              | 1.00             |           |              |                       |          |              |                   |          |              | 1.00             |           |              |
|                   | Blood        | 44.00                  | 34.28-56.47 |              | 0.03             | 0.01-0.08 |              |                       |          |              |                   |          |              | 0.45             | 0.29-0.70 |              |
|                   |              |                        |             | 0.0074       |                  |           |              |                       |          |              |                   |          |              | 0.17             | 0.10-0.27 |              |
| Season            | Summer       | 1.00                   |             |              | 1.00             |           |              |                       |          |              |                   |          |              | 1.00             |           |              |
|                   | Spring       | 1.14                   | 0.87-1.48   |              | 0.66             | 0.52-0.84 |              |                       |          |              |                   |          |              | 1.53             | 1.16-2.02 |              |
|                   | Autumn       | 1.43                   | 1.10-1.85   |              | 0.64             | 0.50-0.81 |              |                       |          |              |                   |          |              | 0.71             | 0.50-1.00 |              |
|                   | Winter       | 0.93                   | 0.71-1.22   |              | 0.83             | 0.65-1.04 |              |                       |          |              |                   |          |              | 1.02             | 0.74-1.40 |              |



and S. I [1],4,[5],12:i:– comprising 16.0% ( $n=316$ ) and 4.5% ( $n=90$ ), of the cases for 2007, respectively (Fig. 1).

### Age distribution

The patients' ages ranged from 1 day to 96 years. The majority of infections ( $n=640$ , 32.6%) were observed in patients between 0 and 5 years (Fig. 1). The frequency of infections among the patients rapidly decreased after the fifth year of life to an average annual level of 3.2–4.4% ( $n=63$ –86) among patients from 6 to 20 years. A higher frequency of infection was also observed among patients in the second and third decades of life. This age group on average represented 144 cases (7.5%) per year. The frequency of infection plateaued through the sixth decade to an average of 60 cases per year (3.1%) at age 60. The frequency of infection peaked for the third time among patients older than 60 years. This age group accounted for an average of 280 cases (14.0%) per year (Table 1, Fig. 2).

### Sex

No differences were observed based on patients' sex. Over the 6-year period, 52.1% of patients were female and 47.9% were male.

### Seasonal trends

Infections peaked in June with an average of 1456 cases (12.5%). Overall, the fewest cases ( $n=745$ ) were reported during the month of April. However, S. I [1],4,[5],12:i:– peaked in April with an average 113 cases compared to 65 cases in June (Table 1, Fig. 3).

### Specimen source

The majority of isolates (82.2%) were recovered from stool or rectal swabs whereas the remaining 17.8% of samples were recovered from blood. Between 2002 and 2005, the percentage of isolates recovered from blood decreased from 15.2% in 2002 to 8.4% in 2005. However, the percentage of blood isolates more than doubled in 2006 to 29.3% and remained relatively unchanged (25.2%) in 2007.

### Regional variation

The majority ( $n=3119$ ) of infections were reported from the Bangkok region (Table 1). The 10 most common serovars described in this study were present in Bangkok; however, the proportion of S. Enteritidis was the lowest (10%) when compared to the other four regions (Fig. 4). A large number of cases ( $n=4057$ ) were also reported in the central region. Similar to Bangkok, all of the serovars described in the study were present in the central region. In addition, the central region had the highest proportion of S. Choleraesuis (11%), which ranked as the sixth most common serovar in that region (Fig. 4).

A large number of infections were also observed in the northern region, specifically within zones 8 and 10. *Salmonella* Kedougou accounted for 5% of the cases in the northern region. In contrast to other regions, S. Panama was not listed among the 10 most commonly reported serovars, having been surpassed by S. Kedougou.

The southern region, which includes both the eastern and the western peninsulas, accounted for the greatest number of salmonellosis cases due to S. Enteritidis (19%) and S. Wel-

tevreden (18%) compared to the other regions. A small percentage of cases in the southern region were due to swine-associated serovars: S. Stanley (8%), S. Rissen (4%), and S. Corvallis (3%). S. Choleraesuis was not among the 10 most commonly reported serovars in the southern region. However, in contrast to other regions, *Salmonella* Albany accounted for 3% of cases and was ranked as the 10th most common serovar in the southern region (Fig. 4).

Only 1228 cases were observed in the northeastern region (zones 5, 6, and 7) and were atypical in comparison with the other regions because neither S. Choleraesuis, S. Corvallis, nor S. Panama were among the top 10 most common serovars (Table 1). These serovars were displaced by S. Hvittingfoss (6%), S. Derby (4%), and S. Virchow (3%) which ranked eighth, ninth, and tenth, respectively, among the top 10 most common serovars in the region (Fig. 4).

### Risk factors

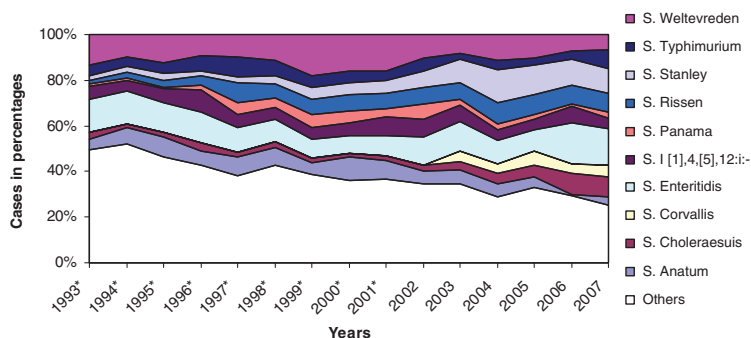
**Blood invasive *Salmonella* serovars.** The statistical analysis revealed that S. Enteritidis, S. Choleraesuis, and to a lesser extent S. I [1],4,[5],12:i:– and S. Typhimurium were recovered from blood at a significantly increased odds ratio when compared to other nontyphoidal *Salmonella* serovars. The highest odds ratio for isolation of S. Choleraesuis or S. Enteritidis from blood was observed among patients older than 5 years, whereas with S. I [1],4,[5],12:i:– the highest odds ratio was observed among patients younger than 6 years. S. Typhimurium was isolated from blood with a higher odds ratio in the southern region (zones 1, 2, 3, and 4); however, age did not appear to be a significant predisposing factor for S. Typhimurium infection.

Seasonal variations were observed among several serovars. The odds ratio for infection with S. Choleraesuis appeared to be higher during autumn (Tables 2 and 3, Fig. 3), while S. Enteritidis cases peaked in winter and S. I [1],4,[5],12:i:– cases increased in spring. Odds ratios for infection with S. Enteritidis and S. I [1],4,[5],12:i:– typically declined during the summer. S. Anatum cases typically increased during summer with a lower odds ratio in the remaining part of the year (Tables 2 and 3, Fig. 3).

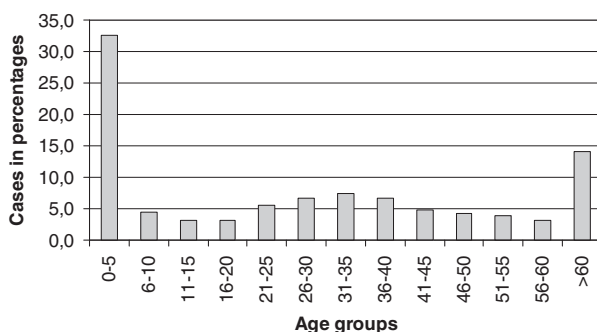
In comparison to the other regions, the highest odds ratio for S. Choleraesuis infection was observed among patients in Bangkok and the central region. Patients in Bangkok and the central region also had the lowest odds ratio of S. I [1],4,[5],12:i:– infections. The highest odds ratio of infection with S. Enteritidis was identified in the southern region. The northeastern region had the lowest rate of S. Panama and S. Corvallis infections (Table 2, Fig. 4).

**Diarrhea-associated *Salmonella* serovars.** The most common *Salmonella* serovars associated with gastrointestinal infections in Thai patients were S. Stanley, S. Weltevreden, S. Rissen, S. Anatum, and S. Corvallis (Table 2). The majority of S. Stanley infections were among patients between 0 and 5 years while S. Weltevreden and S. Anatum primarily infected patients older than 5 years. S. Panama showed a clear association with patients between 0 and 5 years and was typically recovered from stool samples.

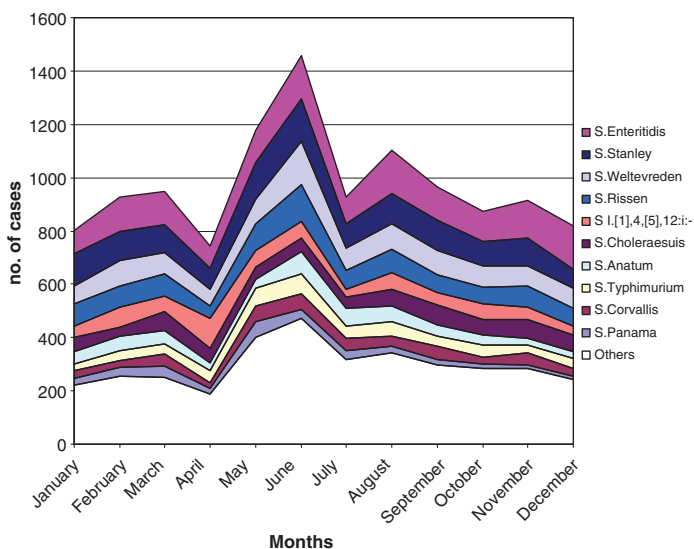
Geographic trends were also observed among these serovars. S. Weltevreden cases were reported predominantly from the southern region and the majority of S. Rissen cases were reported from the northern region and Bangkok



**FIG. 1.** Distribution of the top 10 nontyphoidal *Salmonella* serovars among Thai patients from 1993 to 2007. The top 10 nontyphoidal *Salmonella* serovars as used in the logistic regression analysis. The chart legend lists the different serotypes on the right, which are represented in sequential order in the chart. The percentage of cases for each serovar is represented by the difference in thickness. \*Data include all clinical *Salmonella* isolates.



**FIG. 2.** Distribution of age among Thai patients with salmonellosis from 2002 to 2007.



**FIG. 3.** Seasonal variation of *Salmonella* cases from Thai patients between 2002 and 2007. The chart legend lists the different serotypes on the right, which are represented in sequential order in the chart. The percentage of cases for each serovar is represented by the difference in thickness. The top 10 nontyphoidal *Salmonella* serovars as used in the logistic regression analysis.

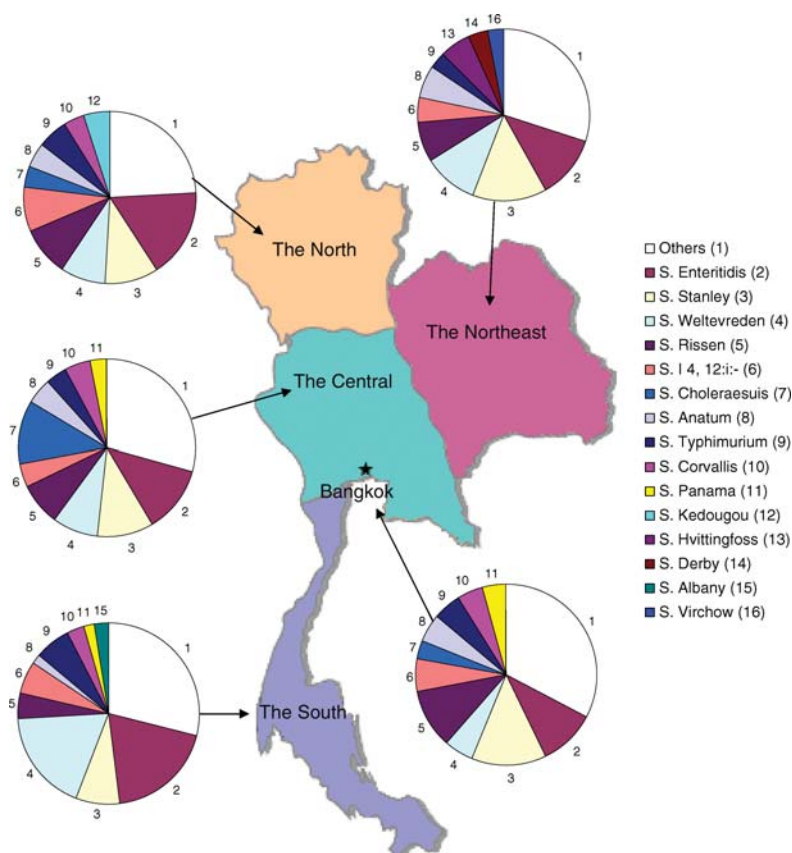


FIG. 4. Distribution of the top 10 most common serovars in the different regions in Thailand from 2002 to 2007. The numbers on the pie charts correspond with the numbered serovars in the chart legend to the right of the figure. Color version of this figure is available online at [www.liebertonline.com](http://www.liebertonline.com).

(Table 2, Fig. 4). The majority of *S. Rissen* cases reported over the course of the study were from the northern region; however, the majority of cases from the northeastern region were due to *S. Stanley* (Table 2 and Fig. 4).

## Discussion

### Occurrence

When compared to previously published studies showing the prevalence from 1993 to 2002, the data in this study suggest a shift in the prevalence of the top 10 nontyphoidal serovars associated with human salmonellosis in Thailand between 2002 and 2007 (Bangtrakulnonth *et al.*, 2004).

Our data indicate that while rates of *S. Weltevreden*, *S. Derby*, *S. Agona*, and *S. Anatum* are decreasing, *S. Stanley*, *S. Choleraesuis*, and *S. Corvallis* are increasing. Although the increases in *S. Corvallis* and *S. Stanley* are notable, the increased occurrence of more invasive serovars such as *S. Choleraesuis* is more worrisome. This increase is particularly concerning amid recent reports of *S. Choleraesuis* strains re-

sistant to fluoroquinolone and extended-spectrum cephalosporin. A recent study of 56 *S. Choleraesuis* isolates reported approximately 60% of the study isolates to be nalidixic acid resistant and 15% to be ceftriaxone resistant (Kulwichit *et al.*, 2007). These reports emphasize the importance of initiating actions to control the spread of this serovar.

The marked decrease in *S. Weltevreden* has made *S. Enteritidis* the most common serovar associated with human infections in Thailand. These data contradict a publication that forecasted a decline in the occurrence of *S. Enteritidis* (Bangtrakulnonth *et al.*, 2004). Interestingly, another study from 2006 failed to find any *S. Enteritidis* in humans from the northern region (Padungtod and Kaneene, 2006).

Previous studies have described possible reservoirs in Thailand for many of the serovars described in this study (Jerngklinchan and Saitanu, 1993; Sakai and Chalermchaikit, 1996; Sasipreeyajan *et al.*, 1996; Bangtrakulnonth *et al.*, 2004; Vaeteewootacharn *et al.*, 2005; Ponce *et al.*, 2008).

Most studies in Thailand have associated *S. Rissen*, *S. Corvallis*, *S. I 1, 4, 5, 12:i:-*, *S. Stanley*, *S. Choleraesuis*, and

*S. Typhimurium* with swine and pork products. In 2004, 295 *S. Rissen* isolates originating from various food sources in Thailand were investigated and the majority ( $n=220$ ) of the isolates originated from pork products (Hendriksen *et al.*, 2008). These results were supported by a study of swine performed in Thailand in 2008; 49% of the tested swine harbored *S. Rissen*. Additionally, 19% and 12% of the animals also harbored *S. Typhimurium* and *S. Stanley*, respectively (Dorn-In *et al.*, 2008).

*S. Corvallis* has also been reported from food sources in Thailand. Publications in 2006 and 2007, described a total of 12 *S. Corvallis* isolates from food sources in Thailand (beef,  $n=2$ ; chicken meat,  $n=6$ ; and pork,  $n=4$ ) (Archambault *et al.*, 2006; Cavaco *et al.*, 2007). The relationship between 138 isolates of *S. Typhimurium* and *S. I* 1, 4, 5, 12:i:– in humans and swine from Thailand has been investigated and swine have been postulated as a reservoir for both serovars (Pornruangwong *et al.*, 2008).

In contrast to the swine-related serovars, *S. Enteritidis*, *S. Anatum*, and *S. Panama* have been associated with chicken and poultry products in Thailand. The most predominant serovar was *S. Enteritidis*, which was present in 28% of retail chicken meat in Thailand (Boonmar *et al.*, 1998a). The same author has also described the spread of a *S. Enteritidis* clone among chickens and humans in Thailand (Boonmar *et al.*, 1998b).

Other studies have also suggested that *S. Panama* may be associated with swine and pork in Thailand, while *S. Rissen*, *S. Corvallis*, and *S. Stanley* may be associated with chicken. A cross-sectional investigation of retail food in Thailand determined that *S. Anatum* was primarily associated with beef and pork while *S. Corvallis* was primarily associated with chicken (Vindigni *et al.*, 2007).

Several studies have also shown an association between *S. Weltevreden* and seafood, environmental sources, or vegetables. However, this serovar has also been found in both swine and chicken. Publications describe *S. Weltevreden* as the most common serovar associated with Thai frozen shrimp (Boonmar *et al.*, 1998c), and it was also found in 22 isolates of 48 isolates recovered from chicken (Padungtod and Kaneene, 2006).

Fermented food has historically been recognized as a potential cause of foodborne disease. Consumption of fermented pork and seafood products is common across Thailand, and contamination with *Salmonella* ssp. has previously been reported with Nham, a traditional Thai fermented ground-pork sausage (Paukatong and Kunawasen, 2001).

Prior to this study, there have only been limited reports of *S. Choleraesuis* in Thailand. Previous reports from Thailand have shown that *S. Choleraesuis* was the second most common cause of septicemia between 1988 and 1996 (Boonmar *et al.*, 1998c; Chiu *et al.*, 2004). Additionally, one article reported the isolation of *S. Choleraesuis* from 54 Thai patients between 2003 and 2005 (Kulwichit *et al.*, 2007).

This study suggests that in the northern region, human infections with swine-associated serovars are increasing and human infections with chicken-associated serovars are decreasing. Conversely, swine-associated serovars are decreasing and chicken- and seafood-associated serovars are increasing in southern Thailand. The distribution of animal-associated serovars may be cultural and could reflect reduced pork consumption and increased poultry consumption within a large Muslim population in southern Thailand.

### Risk factors

Previously published studies have shown that *S. Enteritidis*, *S. Typhimurium*, *S. Panama*, and *S. Choleraesuis* are associated with a higher percentage of extra-intestinal infections and increased hospitalization rates (Helms *et al.*, 2003; Jones *et al.*, 2008). The most commonly recovered serovars from blood samples in Thailand were *S. Enteritidis*, *S. Choleraesuis*, *S. I* [1], 4, [5], 12:i:–, and to a lesser extent *S. Typhimurium* (Tables 2 and 3). Invasive *S. Enteritidis* infections have previously been reported in Thailand. Two publications described increased morbidity associated with *S. Enteritidis* and reported that *S. Enteritidis*, followed by *S. Choleraesuis*, were the most common *Salmonella* serovars recovered from blood (Boriraj *et al.*, 1997; Boonmar *et al.*, 1998c).

Our analysis indicates that patients submitting rectal swabs or stool samples had a greater risk for infection with *S. Stanley*, *S. Weltevreden*, *S. Rissen*, and *S. Anatum* than other nontyphoidal serovars (Tables 2 and 3). Several publications also associated these serovars with gastrointestinal infections. For example, in 2003, *S. Rissen* and *S. Stanley* were the two most common serovars recovered from Thai patients with diarrhea (Angkitittrakul *et al.*, 2005), and *S. Weltevreden* and *S. Anatum* were the leading causes of diarrhea in 1993 to 1996 (Moolasart *et al.*, 1997).

The age distribution of the cases was not unexpected. The majority of the cases occurred among children and older adults. This is consistent with the general understanding of *Salmonella* epidemiology (de Wit *et al.*, 2000). We found that children between 0 and 5 years were at higher risk of being infected with *S. Stanley* as compared to the other nontyphoidal *Salmonella* serovars (Table 2). This is in agreement with data from a previous study in which 4 out of 15 children were infected with *S. Stanley* (Moolasart *et al.*, 1997; Padungtod and Kaneene, 2006).

*S. Anatum* has been reported as a cause of infections among 38% ( $n=32$ ) of crop farm workers (Padungtod and Kaneene, 2006). This result is also in concordance with our analysis that shows a higher risk for *S. Anatum* infection among those older than 6 years.

Patients older than 6 years were also at a greater risk for infection with *S. Enteritidis* and *S. Weltevreden*. These findings are consistent with previous studies (Boriraj *et al.*, 1997). As these serovars have been associated with specific reservoirs, their association with specific age groups may reflect an individual's diet. *S. Weltevreden* for example has been associated with shrimp, a food product not commonly given to very small children (Boonmar *et al.*, 1998c).

Seasonal variation was observed for most cases in the summer period (Fig. 3), which is in agreement with what has been observed elsewhere in the same region (Cho *et al.*, 2008).

The majority of cases were reported from Bangkok and the central region (Fig. 2). This large number of cases reported from Bangkok is most likely due to a combination of population density, better reporting, and the high prevalence of street vendors selling "ready to eat food." The large number of cases from zone 4 (within the central region) may simply be due to logistics, because this area is in close geographic proximity to Bangkok and samples can easily be collected and transported. Reasons for the high proportion of cases in the surrounding districts are uncertain.

We expected to find more human cases in the northern region based on previous studies where 19% of a total of 403 people sampled tested positive for *Salmonella* spp. among the healthy population in the northeastern region (Vaeteewoontacharn *et al.*, 2005). In another study from the northern region the prevalence in livestock farmers and slaughterhouse workers was 36% and 25%, respectively (Padungtod and Kaneene, 2006). It could be speculated that high prevalence is associated with working with animals and that the general population has a much lower prevalence.

The data in this study reveal a high number of infections caused by nontyphoidal *Salmonella* serovars in Thailand, which are potentially preventable. In order to address the problem, the responsible authorities in Thailand need to take action to diminish the level of this burden. It is suggested that a source attribution program be created to reveal the true link between the different *Salmonella* serovars. The information obtained from this attribution study can then be used to control these infections by launching targeted, serovar-specific interventions against the true reservoirs.

#### Limitations

The epidemiological data from Thailand were based on a passive monitoring of samples submitted to the WHO International *Salmonella* and *Shigella* Centre, National Institute of Health.

Throughout the course of the study, three zones (7, 9, and 11) consistently reported low numbers of cases (data not shown). It is not known whether this reflects actual numbers or possible data gaps. The authors do not have specific knowledge of the capacity for clinical laboratories in these zones to isolate *Salmonella* spp. nor do we have any information on the local infrastructure needed for a laboratory-based surveillance system. In addition, habits and local routines may differ from one part of a country to another with regard to how often a doctor is consulted.

#### Conclusion

The outcome of this study indicates a shift in prevalence of the most common *Salmonella* serovars responsible for human infections in Thailand compared to previous studies. Swine-related serovars in Thailand such as *S. Stanley*, *S. Corvallis*, and *S. Choleraesuis* are increasing while *S. Weltevreden* and *S. Anatum* are decreasing. Most infections among Thai patients occur in the age groups 0–5, 31–35, and >60 years. The majority of cases are reported from Bangkok and surrounding areas with a peak in reported infections during the summer months.

Multiple risk factors, which varied by region and serovar, were identified in this study. Based on the variability of these risk factors, the authors recommend initiating interventions targeted to specific serovars taking regional, age group, and seasonal variation into account. We strongly encourage the Thai authorities to initiate interventions against the two main invasive serovars, *S. Enteritidis* and *S. Choleraesuis*. Interventions for *S. Enteritidis* should be focused on patients older than 5 years in the southern region and interventions for *S. Choleraesuis* should be directed towards people older than 5 years in the central region. In addition, studies can be undertaken to elucidate the reasons for the increase in swine-

associated serovars and identify control methods to reduce the burden of disease associated with these organisms. We also suggest initiating longer term prevention and control measures by exploring possibilities to collect data for source attribution focusing on the reservoirs contributing to salmonellosis among the Thai population.

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#### Disclosure Statement

No competing financial interests exist.

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